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(11) Publication number : **0 502 812 A1**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number : **92810056.9**

(22) Date of filing : **27.01.92**

(51) Int. Cl.<sup>5</sup> : **C12N 15/13, C12P 21/08,  
C12N 1/21, G01N 33/574,  
A61K 39/395**

(30) Priority : **05.02.91 EP 91810079**

(43) Date of publication of application :  
**09.09.92 Bulletin 92/37**

(84) Designated Contracting States :  
**AT BE CH DE DK ES FR GB GR IT LI LU NL PT  
SE**

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(54) **Recombinant antibodies specific for a growth factor receptor.**

(57) The invention concerns recombinant antibodies directed to the extracellular domain of the human growth factor receptor c-erbB-2 comprising a light chain variable domain and a heavy chain variable domain of a monoclonal antibody, monoclonal antibodies directed to c-erbB-2 themselves, a method of manufacture of said recombinant antibodies and said monoclonal antibodies, hybridoma cells secreting said monoclonal antibodies, a method of manufacture of said hybridoma cells, DNA coding for the heavy chain variable domain, for the light chain variable domain and for the recombinant antibody, a method of manufacture of said DNA, hybrid vectors suitable for expression of said DNA, host cells transformed with said DNA, and the use of said recombinant antibodies and said monoclonal antibodies in the diagnosis and treatment of tumors.

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Background of the invention

Growth factors and their receptors are involved in the regulation of cell proliferation, and they also seem to play a role in tumor growth. The c-erbB-2 growth factor receptor protein, a protein of the membrane receptor protein tyrosine kinase family (A. Ullrich & J. Schlessinger, Cell 61: 203-212, 1990), is found in human breast tumors and human ovarian carcinomas. Amplification of the c-erbB-2 gene and over-expression of the protein appears to correlate with poor prognosis for tumor patients. Thus the c-erbB-2 protein has potential, both as a diagnostic marker and as a target for cancer therapy. Sequence analysis reveals that c-erbB-2, also called HER2, a glycoprotein of 185 kilo-Dalton (gp 185), is identical or closely related to the human analog of the neu oncogene (A.L. Schechter et al., Science 229: 976-978, 1985) and shows considerable sequence homology to the human epidermal growth factor (EGF) receptor.

Of particular interest in tumor diagnosis and therapy are antibodies directed to tumor markers. Polyclonal antibodies may be obtained from the serum of mammals immunized with the antigen, i.e. the tumor marker. The development of hybridoma technology made it possible to generate continuous cell lines, in particular murine hybridomas, producing monoclonal antibodies of the desired specificity. Murine monoclonal antibodies directed to c-erbB-2 are known and are described, for example, by S.J. McKenzie et al., Oncogene 4: 543-548, 1989; R.M. Hudziak et al., Molecular and Cellular Biology 9: 1165-1172, 1989; International Patent Application WO 89/06692 (Genentech); and Japanese Patent Application Kokai 02-150 293 (Ajinomoto KK).

A major limitation in the use of murine-derived monoclonal antibodies as in vivo diagnostic and therapeutic agents is their immunogenicity as foreign proteins, their rather long persistence in the circulation, and the formation of damaging immune complexes. On the other hand, the treatment with human monoclonal antibodies is also limited since human hybridoma cell lines are hard to prepare, generally unstable, and do not produce monoclonal antibodies of appropriate specificity in sufficient quantities and at reasonable costs. In principle, the in vitro use of murine monoclonal antibodies is without limitation. However, production costs of monoclonal antibodies and, depending on the type of immunoassay used, the need for attaching a detectable marker to the antibody make it desirable to find more economic alternatives to regular murine monoclonal antibodies.

A promising alternative is the modification of immunoglobulin genes in order to tailor antibodies for particular diagnostic and therapeutic tasks. Due to the fact that the variable region and each of the constant region domains of immunoglobulin molecules are encoded in separate exons with their own splice sites, recombinant DNA techniques can be used to isolate different parts of cloned immunoglobulin genes and ligate them to parts of other immunoglobulins or to effector molecules. The reconstructed genes are expressed by appropriate transformed continuous cell lines. Murine antibodies can, for example, be converted into "humanized" antibodies by exchanging murine constant domain exons for human immunoglobulin constant domain exons, thus generating chimeric antibodies with murine antibody-combining sites and human constant domains. The chimeric antibodies retain the antigen specificity determined by the murine variable domains, but also exhibit human effector functions (such as complement binding, stimulation of phagocytosis, triggering of granule release by mast cells) determined by the carboxy-terminal constant domain segments of the heavy chain polypeptides. An even more sophisticated technique in tailoring antibodies described in European Patent Application 0 239 400 exchanges also other fairly conserved domains, the so-called framework regions (FRs), within the murine variable domains for corresponding framework regions from human antibodies or for other human protein sequences. Such an antibody should be even less immunogenic in man since the only parts derived from a murine antibody are those hypervariable regions which define a particular specificity for an antigen; the so-called complementarity determining regions (CDRs).

Furthermore, fusion proteins different from immunoglobulins may be formed, e.g. single-chain antibodies, which retain the specificity and binding properties of the starting murine monoclonal antibody, but have otherwise novel properties derived from the non-immunoglobulin part of the fusion protein. The smallest domain of a monoclonal antibody which can bind to the antigen is the so-called Fv fragment which consists of the variable domains of the heavy and light chains. Fv fragments are difficult to prepare by proteolytic techniques since the corresponding variable domains tend to dissociate upon dilution. Fv molecules constructed by joining the variable domains of the heavy and light chains via a short peptide linker, also called single-chain antigen binding proteins, bind to an antigen with similar characteristics as the original monoclonal antibody (R.E. Bird et al., Science 242: 423-426, 1988; J.S. Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879-5883, 1988; and International Patent Application WO 89/09825 (Celltech)). Fv encoding genes can, in principle, be linked to genes encoding effector molecules by recombinant gene technology. It is known, for example, that Fv encoding gene sequences can be linked to a gene encoding a portion of the *Pseudomonas* exotoxin A gene (V.K. Chaudhary et al., Nature 339: 394-397, 1989; and International Patent Application WO 89/11533 (I. Pastan et al.)).

Object of the invention

It is an object of this invention to provide recombinant antibodies directed to the extracellular domain of the human growth factor receptor c-erbB-2 comprising a light chain variable domain and a heavy chain variable domain of a monoclonal antibody, monoclonal antibodies directed to c-erbB-2 themselves, a method of manufacture of said recombinant antibodies and said monoclonal antibodies, hybridoma cells secreting said monoclonal antibodies, a method of manufacture of said hybridoma cells, DNA coding for the heavy chain variable domain, for the light chain variable domain and for the recombinant antibody, a method of manufacture of said DNA, hybrid vectors suitable for expression of said DNA, host cells transformed with said DNA, and the use of said recombinant antibodies and said monoclonal antibodies in the diagnosis and treatment of tumors.

Detailed description of the invention

The invention concerns a recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, a human glycoprotein of 185 kilo-Dalton (gp185), comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody.

Such a recombinant antibody may be a chimeric antibody consisting, for example, of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human heavy chain constant domain  $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , or  $\mu$ , preferably  $\gamma$ , such as  $\gamma 1$  or  $\gamma 4$ , and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain  $\kappa$  or  $\lambda$ , preferably  $\kappa$ , all assembled to give a functional antibody.

The preferred recombinant antibody of the invention is a single-chain antibody wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group, preferably a peptide. Most preferred is a single-chain antibody wherein the heavy chain variable domain is located at the N-terminus of the recombinant antibody. The single-chain recombinant antibody may further comprise an effector molecule and/or signal sequences facilitating the processing of the antibody by the host cell in which it is prepared. Effector molecules considered are those useful for diagnostic or therapeutic purposes, for example enzymes causing a detectable reaction, e.g. phosphatase, such as alkaline phosphatase from *E. coli* or mammalian alkaline phosphatase, e.g. bovine alkaline phosphatase, horseradish peroxidase,  $\beta$ -D-galactosidase, glucose oxidase, glucoamylase, carbonic anhydrase, acetylcholinesterase, lysozyme, malate dehydrogenase or glucose-6-phosphate, a peptide having particular binding properties, e.g. streptavidin from *Streptomyces avidinii* strongly binding to biotin, or enzymes, toxins or other drugs attacking the cells to which the antibody is bound, e.g. a protease, a cytolytic or an exotoxin, for example ricin A, diphtheria toxin A, or *Pseudomonas* exotoxin. In the following a single-chain recombinant antibody further comprising an effector molecule is referred to as fusion protein or intended to be within the meaning of the terms "single chain (recombinant) antibody" or "recombinant antibody", if appropriate.

The term effector molecule also includes biologically active variants of the above-mentioned proteins, e.g. variants produced from a DNA which has been subjected to *in vitro* mutagenesis, with the provision that the protein encoded by said DNA retains the biological activity of the native protein. Such modifications may consist in an addition, exchange or deletion of amino acids, the latter resulting in shortened variants. For example, an enzyme, such as phosphatase, may be prepared from a DNA which has been modified to facilitate the cloning of the encoding gene, or an exotoxin, such as *Pseudomonas* exotoxin, may be prepared from a DNA which has been mutated to delete the cell binding domain.

The recombinant antibodies of the invention are tested for their specificity to the extracellular domain of c-erbB-2, for example by immunofluorescent staining of cells expressing high levels of c-erbB-2, by immunoblotting either directly or by way of immunoprecipitation and protein blotting of the immunocomplexes, or by another immunoassay such as a binding, crossinhibition or competition radio- or enzyme immunoassay.

The variable domain of an antibody heavy or light chain consists of so-called framework regions (FRs), which are fairly conserved in antibodies with different specificities, and of hypervariable regions also called complementarity determining regions (CDRs), which are typical for a particular specificity.

Preferred recombinant antibodies of the invention are those wherein the heavy chain variable domain comprises a polypeptide of the formula

$$FR_1-CDR_{1H}-FR_2-CDR_{2H}-FR_3-CDR_{3H}-FR_4 \quad (I)$$

wherein  $FR_1$  is a polypeptide residue comprising at least 25-29, preferably 25-33 naturally occurring amino acids,  $FR_2$  is a polypeptide residue comprising 12-16 naturally occurring amino acids,  $FR_3$  is a polypeptide residue comprising 30-34 naturally occurring amino acids,  $FR_4$  is a polypeptide residue comprising at least 6-10, preferably 6-13 naturally occurring amino acids,  $CDR_{1H}$  is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4,  $CDR_{2H}$  is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4, and  $CDR_{3H}$  is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4, or,  $CDR_{1H}$  is a

p polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:8, CDR<sub>2H</sub> is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:8, and CDR<sub>3H</sub> is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges. These particular complementarity determining regions are Asn-Tyr-Gly-Met-Asn (CDR<sub>1H</sub>), Trp-Ile-Asn-Thr-Ser-Thr-Gly-Glu-Ser-Thr-Phe-Ala-Asp-Asp-Phe-Lys-Gly (CDR<sub>2H</sub>), and Trp-Glu-Val-Tyr-His-Gly-Tyr-Val-Pro-Tyr (CDR<sub>3H</sub>) according to SEQ. ID NO: 4, or Ser-Tyr-Trp-Met-Asn (CDR<sub>1H</sub>), Met-Ile-Asp-Pro-Ser-Asp-Ser-Glu-Thr-Gln-Tyr-Asn-Gln-Met-Phe-Lys-Asp (CDR<sub>2H</sub>) and Gly-Gly-Ala-Ser-Gly-Asp-Trp-Tyr-Phe-Asp-Val (CDR<sub>3H</sub>) according to SEQ. ID NO:8.

Especially preferred are recombinant antibodies comprising a heavy chain variable domain of formula I, wherein the polypeptide residues of the framework regions FR<sub>1</sub>, FR<sub>2</sub>, FR<sub>3</sub> and FR<sub>4</sub> are those preferably occurring in mammalian, especially murine or human, antibodies.

In a first embodiment of the invention, most preferred are recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 120, of SEQ ID NO:4, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 2 to 31 (FR<sub>1</sub>), 37 to 50 (FR<sub>2</sub>), 68 to 99 (FR<sub>3</sub>), and/or 110 to 120 (FR<sub>4</sub>), are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

In a second embodiment of the invention, most preferred are recombinant antibodies wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121, of SEQ ID NO: 8, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 2 to 31 (FR<sub>1</sub>), 37 to 50 (FR<sub>2</sub>), 68 to 99 (FR<sub>3</sub>), and/or 111 to 121 (FR<sub>4</sub>), are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a heavy chain variable domain comprising a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO: 8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

For example, a hydrophobic amino acid within the framework regions may be replaced by another amino acid, preferably also a hydrophobic amino acid, e.g. a homologous amino acid, replaced by two amino acids, or deleted. Likewise, a hydrophilic amino acid within the framework region may be replaced by another amino acid, two amino acids or deleted, whereby replacing amino acids preferably maintain the hydrogen bond structure of the corresponding framework region.

Likewise preferred recombinant antibodies of the invention are those wherein the light chain variable domain comprises a polypeptide of the formula



wherein FR<sub>5</sub> is a polypeptide residue comprising naturally occurring amino acids, preferably 19-25, especially 19-23 naturally occurring amino acids, FR<sub>7</sub> is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR<sub>8</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>9</sub> is a polypeptide residue comprising naturally occurring amino acids, particularly 7-11 naturally occurring amino acids, and CDR<sub>1L</sub> is a polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4, CDR<sub>2L</sub> is a polypeptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4, and CDR<sub>3L</sub> is a polypeptide residue of the amino acid sequence 224 to 232 of SEQ ID NO:4, or CDR<sub>1L</sub> is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:8, CDR<sub>2L</sub> is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO:8, and CDR<sub>3L</sub> is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges. These particular complementarity determining regions are Lys-Ala-Ser-Gln-Asp-Val-Tyr-Asn-Ala-Val-Ala (CDR<sub>1L</sub>), Ser-Ala-Ser-Ser-Arg-Tyr-Thr (CDR<sub>2L</sub>), and Gln-Gln-His-Phe-Arg-Thr-Pro-Phe-Thr (CDR<sub>3L</sub>) according to SEQ ID No:4, or Lys-Ala-Ser-Gln-Asp-Ile-Lys-Lys-Tyr-Ile-Ala (CDR<sub>1L</sub>), Tyr-Thr-Ser-Val-Leu-Gln-Pro (CDR<sub>2L</sub>) and Leu-His-Tyr-Asp-Tyr-Leu-Tyr-Thr (CDR<sub>3L</sub>) according to SEQ ID No. 8.

Especially preferred are recombinant antibodies comprising a light chain variable domain of formula II, wherein the polypeptide residues of the framework regions FR<sub>5</sub>, FR<sub>6</sub>, FR<sub>7</sub> and FR<sub>8</sub> are those preferably occurring in mammalian, especially murine or human, antibodies.

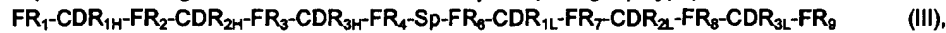
In one embodiment of the invention, most preferred are recombinant antibodies wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein optionally one or more, e.g. 1, 2, 3 or 4, single amino acids within the amino acid sequences 136 to 158 (FR<sub>5</sub>), 170 to 184 (FR<sub>6</sub>), 192 to 223 (FR<sub>7</sub>), and/or 233 to 241 (FR<sub>8</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibodies with a light chain variable domain comprising a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

In a second embodiment of the invention, most preferred are recombinant antibodies wherein the light chain

variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein optionally one or more, e.g. 1, 2, 3 or 4 single amino acids within the amino acid sequences 137 to 159 (FR<sub>6</sub>), 171 to 185 (FR<sub>7</sub>), 193 to 224 (FR<sub>8</sub>), and/or 233 to 241 (FR<sub>9</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges, in particular the recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

For example, amino acids within the framework regions may be replaced by other amino acids or deleted as detailed above for the heavy chain.

Especially preferred is a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are linked by way of a spacer group consisting of 10 to 30, e.g. around 15, amino acids, in particular a single-chain recombinant antibody comprising a polypeptide of the formula



wherein FR<sub>1</sub>, CDR<sub>1H</sub>, FR<sub>2</sub>, CDR<sub>2H</sub>, FR<sub>3</sub>, CDR<sub>3H</sub>, FR<sub>4</sub>, FR<sub>5</sub>, CDR<sub>1L</sub>, FR<sub>7</sub>, CDR<sub>2L</sub>, FR<sub>8</sub>, CDR<sub>3L</sub> and FR<sub>9</sub> have the meanings as mentioned before and Sp is a peptide spacer consisting of about 10 to 30, e.g. around 15, amino acids; and wherein the heavy chain or the light chain variable domain is further connected to an effector molecule, e.g. an enzyme, such as phosphatase, particularly alkaline phosphatase, or a toxin, such as Pseudomonas exotoxin, or a variant thereof. Preferably, the effector molecule is connected to the light chain variable domain, optionally via a peptide spacer consisting of one or more, e.g. 1-10 amino acids.

These fusion proteins comprising a single-chain recombinant antibody and an effector molecule optionally comprise another peptide, e.g. a peptide facilitating purification, in particular a peptide being an epitope against which an antibody is available, such as the FLAG peptide. Purification, e.g. by means of affinity chromatography, of a fusion protein comprising such a peptide is advantageous e.g. in that it may be faster, more specific and/or gentler. The peptide may be placed at the N-terminus of the fusion protein, in between the recombinant antibody and the effector molecule, or at the C-terminus of the fusion protein. Preferably, it is located at the N-terminus or at the C-terminus, in particular at the N-terminus. Preferably, these constructs also contain a cleavage site, so that the fusion protein can be liberated therefrom, either by enzymatic cleavage, e.g. by enterokinase or by Factor Xa, or by the chemical methods known in the art. Furthermore these constructs may comprise a peptide spacer consisting of one or more, e.g. 1 to 10, in particular about 2 amino acids, said spacer facilitating the linkage of the above-mentioned peptide and/or the cleavage site to the recombinant antibody. The cleavage site is placed in such a way that the fusion protein comprising the recombinant antibody and the effector molecule can be easily liberated, if desired, preferably in vitro. For example, in the protein construct comprising the fusion protein designated Fv(FRP5)-ETA (cf. SEQ. ID NO: 10), the FLAG peptide and an enterokinase cleavage site are linked to a spacer and placed in front of the Fv heavy chain/light chain variable domain and exotoxin A fusion protein. If desired, the FLAG peptide can be cleaved off by enterokinase, preferably after affinity purification of the protein, yielding a fusion protein comprising the single-chain antibody Fv(FRP5) and exotoxin A.

Most preferred is a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derived from a mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, e.g. derived from the mouse monoclonal antibodies FRP5, FSP16, FWP51 or FSP77, particularly from the mouse monoclonal antibodies FRP5 or FWP51. Likewise preferred is a single-chain recombinant antibody wherein the spacer group linking the light chain and the heavy chain variable domains is a polypeptide comprising about 15 amino acids selected from glycine and serine, in particular wherein the spacer group is the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Ser.

Especially preferred is a single-chain antibody comprising the heavy chain variable domain of a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77, the 15 amino acid spacer group consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77 and an enzyme, for example a phosphatase such as the alkaline phosphatase phoA, or an exotoxin such as Pseudomonas exotoxin, or a variant thereof.

Particularly preferred is the particular single-chain recombinant antibody designated Fv(FRP5)-phoA comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO: 5.

Likewise preferred is a single-chain recombinant antibody comprising a peptide facilitating purification, a cleavage site and a particular single-chain recombinant antibody selected from the group consisting of Fv(FRP5)-ETA and Fv(FWP51)-ETA, in particular a single-chain recombinant antibody comprising a polypeptide selected from the group consisting of a polypeptide of the amino acid sequence -10 to 606 of SEQ. ID NO: 10 and of a polypeptide of the amino acid sequence -10 to 606 of SEQ. ID NO: 11, said protein being subjected to in vitro cleavage by enterokinase, if desired.

Particularly preferred is a single-chain recombinant antibody comprising a protein selected from the group consisting of a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 10 and a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 11.

The invention further concerns the mouse monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 and designated FRP5, FSP16, FSP77, and FWP51, which are secreted by the hybridoma cell lines FRP5, FSP16, FSP77, and FWP51, respectively. Most preferred are the mouse monoclonal antibodies designated FRP5 and FWP51.

The invention further concerns a method of manufacture of the recombinant antibodies and of the mouse monoclonal antibodies of the invention. The antibodies are prepared by processes that are known *per se*, characterized in that host cells or hybridoma cells as defined further below producing such antibodies are multiplied *in vitro* or *in vivo* and, when required, the obtained antibodies are isolated. For example, the recombinant antibodies of the invention can be prepared by recombinant DNA techniques comprising culturing a transformed host under conditions which allow expression thereof and isolating said antibody.

More specifically, the present invention also relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. *E. coli*, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter and a DNA coding for said protein which DNA is controlled by said promoter, and isolating said protein.

In particular, the present invention relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. *E. coli*, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein.

Multiplication of hybridoma cells or mammalian host cells *in vitro* is carried out in suitable culture media, which are the customary standard culture media, for example Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. fetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

*In vitro* production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells *in vivo*. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to two weeks, ascitic fluid is taken from the animals.

The cell culture supernatants are screened for the desired antibodies, preferentially by immunofluorescent staining of cells expressing c-erbB-2, by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-)affinity chromatography, e.g. affinity

chromatography with c-erbB-2 protein or with Protein-A.

The invention further concerns hybridoma cells secreting the monoclonal antibodies of the invention, in particular the hybridoma cell lines FRP5, FSP16, FSP77, and FWP51 deposited under the Budapest Treaty on November 21, 1990 at the European Collection of Animal Cell Cultures (ECACC) in Porton Down, Salisbury, UK, under the accession numbers 90112115, 90112116, 90112117, and 90112118, respectively. Most preferred is the hybridoma cell line designated FRP5, ECACC number 90112115 or the hybridoma cell line designated FWP51, ECACC number 90112118. The preferred hybridoma cells of the invention are genetically stable, secrete monoclonal antibodies of the invention of the desired specificity and can be activated from deep-frozen cultures by thawing and recloning.

The invention also concerns a process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2, characterized in that a suitable mammal, for example a Balb/c mouse, is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing growth factor receptor c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected. For example spleen cells of Balb/c mice immunized with cells bearing c-erbB-2 are fused with cells of the myeloma cell line PA1 or the myeloma cell line Sp2/O-Ag 14, the obtained hybrid cells are screened for secretion of the desired antibodies, and positive hybridoma cells are cloned.

Preferred is a process for the preparation of a hybridoma cell line, characterized in that Balb/c mice are immunized by injecting subcutaneously and/or intraperitoneally between  $10^7$  and  $10^8$  cells of the human breast tumor cell line SKBR3 containing a suitable adjuvant several times, e.g. four to six times, over several months, e.g. between two and four months, and spleen cells from the immunized mice are taken two to four days after the last injection and fused with cells of the myeloma cell line PA1 in the presence of a fusion promoter, preferably polyethylene glycol. Preferably the myeloma cells are fused with a three- to twentyfold excess of spleen cells from the immunized mice in a solution containing about 30 % to about 50 % polyethylene glycol of a molecular weight around 4000. After the fusion the cells are expanded in suitable culture media as described hereinbefore, supplemented with a selection medium, for example HAT medium, at regular intervals in order to prevent normal myeloma cells from overgrowing the desired hybridoma cells.

The invention also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 as described hereinbefore. By definition such DNAs comprise coding single stranded DNAs, double stranded DNAs consisting of said coding DNAs and of complementary DNAs thereto, or these complementary (single stranded) DNAs themselves.

Furthermore, DNA encoding a heavy chain murine variable domain and/or for a light chain murine variable domain of antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 can be enzymatically or chemically synthesized DNA having the authentic DNA sequence coding for a heavy chain murine variable domain and/or for the light chain murine variable domain, or a mutant thereof. A mutant of the authentic DNA is a DNA encoding a heavy chain murine variable domain and/or a light chain murine variable domain of the above-mentioned antibodies in which one or more amino acids are deleted or exchanged with one or more other amino acids. Preferably said modification(s) are outside the CDRs of the heavy chain murine variable domain and/or of the light chain murine variable domain of the antibody. Such a mutant DNA is also intended to be a silent mutant wherein one or more nucleotides are replaced by other nucleotides with the new codons coding for the same amino acid(s). Such a mutant sequence is also a degenerated sequence. Degenerated sequences are degenerated within the meaning of the genetic code in that an unlimited number of nucleotides are replaced by other nucleotides without resulting in a change of the amino acid sequence originally encoded. Such degenerated sequences may be useful due to their different restriction sites and/or frequency of particular codons which are preferred by the specific host, particularly *E. coli*, to obtain an optimal expression of the heavy chain murine variable domain and/or a light chain murine variable domain.

The term mutant is intended to include a DNA mutant obtained by in vitro mutagenesis of the authentic DNA according to methods known in the art.

The invention relates to a recombinant DNA comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of the antibodies FRP5, FSP16, FSP77 and FWP51, or coding for an amino acid sequence homologous to said heavy chain variable domain.

In particular, the invention concerns a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell lines FRP5, FSP16, FSP77 or FWP51, or which is homologous to genomic DNA of said cell lines and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibodies FRP5, FSP16, FSP77 or FWP51. Especially preferred is a recombinant DNA comprising an insert coding for a heavy chain murine

variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FRP5, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibody FRP5; or a recombinant DNA comprising an insert coding for a heavy chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FWP51, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the heavy chain variable domain of monoclonal antibody FWP51.

Preferred is a recombinant DNA comprising an insert coding for the polypeptide of formula I, wherein FR<sub>1</sub>, FR<sub>2</sub>, FR<sub>3</sub>, FR<sub>4</sub>, CDR<sub>1H</sub>, CDR<sub>2H</sub>, and CDR<sub>3H</sub> have the meanings as mentioned hereinbefore, optionally further containing introns. Especially preferred is a recombinant DNA coding for the polypeptide of formula I comprising inserts coding for murine or human framework regions FR<sub>1</sub>, FR<sub>2</sub>, FR<sub>3</sub> and FR<sub>4</sub>, and inserts coding for complementarity determining regions of the DNA sequence 99 to 113 (CDR<sub>1H</sub>), the DNA sequence 156 to 206 (CDR<sub>2H</sub>), and the DNA sequence 303 to 332 (CDR<sub>3H</sub>) of SEQ ID NO:4 or coding for complementarity determining regions of the DNA sequence 99 to 113 (CDR<sub>1H</sub>), the DNA sequence 156 to 206 (CDR<sub>2H</sub>), and the DNA sequence 303 to 335 (CDR<sub>3H</sub>) of SEQ ID NO:8. Most preferred is a DNA comprising an insert of the DNA sequence 9 to 365 of SEQ ID NO:4, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 9 to 365 of SEQ ID NO:4. Likewise preferred is a DNA comprising an insert of the DNA sequence 9 to 368 of SEQ ID NO:8, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 9 to 368 of SEQ ID NO:8.

In a DNA wherein nucleotides of the sequence given in SEQ ID NO:4, or in a DNA wherein nucleotides of the sequence given in SEQ ID NO:8, are replaced by other nucleotides, such replacement is preferred when it does not alter the amino acid sequence of the complementarity determining regions (CDRs) coded for. This means that such replacement of nucleotides may occur in the inserts coding for the framework regions (FRs) or in a position where it does not alter the amino acid coded for due to the degeneracy of the triplet codons.

Likewise the invention relates to a recombinant DNA comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of the antibodies FRP5, FSP16, FSP77 and FWP51, or coding for an amino acid sequence homologous to said light chain variable domain.

More specifically, the invention concerns a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell lines FRP5, FSP16, FSP77 or FWP51, or which is homologous to genomic DNA of said cell lines and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibodies FRP5, FSP16, FSP77 or FWP51. Particularly preferred is a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FRP5, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibody FRP5, or a recombinant DNA comprising an insert coding for a light chain murine variable domain, which originates from genomic DNA or mRNA of the hybridoma cell line FWP51, or which is homologous to genomic DNA of said cell line and codes for an amino acid sequence homologous to the light chain variable domain of monoclonal antibody FWP51.

Preferred is a recombinant DNA comprising an insert coding for the polypeptide of formula II, wherein FR<sub>5</sub>, FR<sub>6</sub>, FR<sub>7</sub>, FR<sub>8</sub>, CDR<sub>1L</sub>, CDR<sub>2L</sub>, and CDR<sub>3L</sub> have the meanings as mentioned hereinbefore, optionally further containing introns. Especially preferred is a recombinant DNA coding for the polypeptide of formula II comprising inserts coding for murine or human framework regions FR<sub>5</sub>, FR<sub>6</sub>, FR<sub>7</sub> and FR<sub>8</sub>, and inserts coding for complementarity determining regions of the DNA sequence 480 to 512 (CDR<sub>1L</sub>), the DNA sequence 558 to 578 (CDR<sub>2L</sub>), and the DNA sequence 675 to 701 (CDR<sub>3L</sub>) of SEQ ID NO:4, or coding for complementarity determining regions of the DNA sequence 483 to 515 (CDR<sub>1L</sub>), the DNA sequence 561 to 581 (CDR<sub>2L</sub>), and the DNA sequence 678 to 701 (CDR<sub>3L</sub>) of SEQ ID NO:8.

Most preferred is a DNA comprising an insert of the DNA sequence 411 to 728 of SEQ ID NO:4, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 411 to 728 of SEQ ID NO:4. Likewise preferred is a DNA comprising an insert of the DNA sequence 414 to 728 of SEQ ID NO:8, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 414 to 728 of SEQ ID NO:8. In a DNA wherein nucleotides of the sequence given in SEQ ID NO:4, or in a DNA wherein nucleotides of the sequence given in SEQ ID NO:8, are replaced by other nucleotides, such replacement is preferred when it does not alter the amino acid sequence of the complementarity determining regions (CDRs) coded for, as is described above for DNA coding for the heavy chain variable domain.

For the assembly of complete tetrameric immunoglobulin molecules and the expression of chimeric antibodies, the recombinant DNA inserts coding for heavy and light chain variable domains are fused with the corresponding DNAs coding for heavy and light chain constant domains, then transferred into appropriate host



cells, for example after incorporation into hybrid vectors.

The invention therefore also concerns recombinant DNAs comprising an insert coding for a heavy chain murine variable domain of an antibody directed to the extracellular domain of c-erbB-2 fused to a human constant domain  $\gamma$ , for example  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$  or  $\gamma 4$ , preferably  $\gamma 1$  or  $\gamma 4$ . Likewise the invention concerns recombinant DNAs comprising an insert coding for a light chain murine variable domain of an antibody directed to the extracellular domain of c-erbB-2 fused to a human constant domain  $\kappa$  or  $\lambda$ , preferably  $\kappa$ .

The invention especially concerns recombinant DNAs coding for a single-chain recombinant antibody as defined hereinbefore, e.g. recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, in particular a recombinant DNA coding for a protein of the formula III, wherein  $FR_1, FR_2, FR_3, FR_4, FR_5, FR_6, FR_7, FR_8, FR_9$ , SP,  $CDR_{1H}, CDR_{2H}, CDR_{3H}, CDR_{1L}, CDR_{2L}$  and  $CDR_{3L}$  have the meanings given above, optionally comprising further DNA coding for an effector molecule and/or signal sequences facilitating the processing of the antibody in the host cell. In particular the invention concerns a DNA comprising an insert of the DNA sequence 9-728 of SEQ ID NO:4, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, especially a DNA comprising an insert of the DNA sequence 9 to 728 of SEQ ID NO:4. Furthermore the invention relates to a DNA comprising an insert of the DNA sequence 9-728 of SEQ ID NO:8 wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, especially a DNA comprising an insert of the DNA sequence 9 to 728 of SEQ ID NO:8.

In another embodiment the invention pertains to recombinant DNAs coding for a recombinant DNA wherein the heavy chain variable domain and the light chain variable domain are linked by way of a DNA insert coding for a spacer group, optionally comprising a signal sequence facilitating the processing of the antibody in the host cell and/or a DNA coding for a peptide facilitating the purification of the antibody and/or a DNA coding for a cleavage site and/or a DNA coding for a peptide spacer and/or a DNA coding for an effector molecule.

The DNA coding for an effector molecule is intended to be a DNA coding for the above-mentioned effector molecules, particularly a DNA coding for alkaline phosphatase or Pseudomonas exotoxin A. The DNA encoding such an effector molecule has the sequence of a naturally occurring enzyme or toxin encoding DNA, or a mutant thereof, and can be prepared by methods well known in the art. A mutant of the naturally occurring DNA encoding e.g. alkaline phosphatase or Pseudomonas exotoxin A, or a variant thereof can be obtained e.g. analogously to the methods described above.

Most preferred is a DNA comprising an insert of the DNA sequence 23 to 814 of SEQ ID NO:5, of the DNA sequence 86 to 2155 of SEQ ID NO:5 or of the DNA sequence 23 to 2155 of SEQ ID NO:5, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 23 to 2155 of SEQ ID NO:5.

Equally preferred is a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 10, of the DNA sequence 64 to 1911 of SEQ ID NO: 10, or of the DNA sequence 97 to 1911 of SEQ ID NO: 10, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 10; or a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 11, of the DNA sequence 64 to 1911 of SEQ ID NO: 11, of the DNA sequence 96 to 1911 of SEQ ID NO: 11, or of the DNA sequence 97 to 1911 of SEQ ID NO: 11, wherein optionally one or more, e.g. 1 to 10, nucleotides are replaced by other nucleotides, in particular a DNA comprising an insert of the DNA sequence 1 to 1911 of SEQ ID NO: 11.

Furthermore the invention concerns a recombinant DNA which is a hybrid vector comprising an insert coding for the variable domain of a murine heavy chain as described hereinbefore and/or an insert coding for the variable domain of a murine light chain as described hereinbefore, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.

In a first embodiment the hybrid vector according to the invention comprises an expression cassette comprising a promoter and a DNA coding for a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer, which DNA is controlled by said promoter, and isolating said protein.

In a second embodiment, the hybrid vector according to the invention comprises an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, and a fusion protein optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

Vectors typically perform two functions in collaboration with compatible host cells. One function is to facilitate the cloning of the nucleic acid that encodes the immunoglobulin variable domains, i.e. to produce usable quantities of the nucleic acid (cloning vectors). The other function is to provide for replication and expression of the recombinant gene constructs in a suitable host, either by maintenance as an extrachromosomal element or by integration into the host chromosome (expression vectors). A cloning vector comprises the recombinant gene constructs as described above, an origin of replication or an autonomously replicating sequence, dominant marker sequences and, optionally, signal sequences and additional restriction sites. An expression vector additionally comprises expression control sequences essential for the transcription and translation of the recombinant genes.

An origin of replication or an autonomously replicating sequence is provided either by construction of the vector to include an exogenous origin such as derived from Simian virus 40 (SV 40) or another viral source, or by the host cell chromosomal mechanisms.

The markers allow for selection of host cells which contain the vector. Selection markers include genes which confer resistance to heavy metals such as copper or to antibiotics such as geneticin (G-418) or hygromycin, or genes which complement a genetic lesion of the host cell such as the absence of thymidin kinase, hypoxanthine phosphoryl transferase, dihydrofolate reductase or the like.

Signal sequences may be, for example, presequences or secretory leaders directing the secretion of the recombinant antibody, splice signals, or the like. Examples for signal sequences directing the secretion of the recombinant antibody are sequences derived from the *ompA* gene, the *pelB* (pectate lyase) gene or the *phoA* gene.

As expression control sequences, the vector DNA comprises a promoter, sequences necessary for the initiation and termination of transcription and for stabilizing the mRNA and, optionally, enhancers and further regulatory sequences.

A wide variety of promoting sequences may be employed, depending on the nature of the host cell. Promoters that are strong and at the same time well regulated are the most useful. Sequences for the initiation of translation are for example Shine-Dalgarno sequences. Sequences necessary for the initiation and termination of transcription and for stabilizing the mRNA are commonly available from the noncoding 5'-regions and 3'-regions, respectively, of viral or eukaryotic cDNAs, e.g. from the expression host. Enhancers are transcription-stimulating DNA sequences of viral origin, e.g. derived from Simian virus, polyoma virus, bovine papilloma virus or Moloney sarcoma virus, or of genomic, especially murine, origin.

The various DNA segments of the vector DNA are operationally linked, i.e. they are contiguous and placed into a functional relationship with each other.

Examples of vectors which are suitable for replication and expression in an *E. coli* strain are bacteriophages, for example derivatives of  $\lambda$  bacteriophages, or plasmids, such as, in particular, the plasmid ColE1 and its derivatives, for example pMB9, pSF2124, pBR317 or pBR322 and plasmids derived from pBR322, such as pUC9, pUCK0, pHR148 and pLc24. Suitable vectors contain a complete replicon, a marker gene, recognition sequences for restriction endonucleases, so that the foreign DNA and, if appropriate, the expression control sequence can be inserted at these sites, and optionally signal sequences and enhancers.

Microbial promoters are, for example, the strong leftward promoter  $P_L$  of bacteriophage  $\lambda$  which is controlled by a temperature sensitive repressor. Also suitable are *E. coli* promoters such as the *lac* (lactose) promoter regulated by the *lac* repressor and induced by isopropyl- $\beta$ -D-thiogalactoside, the *trp* (tryptophan) promoter regulated by the *trp* repressor and induced e.g. by tryptophan starvation, and the *tac* (hybrid *trp-lac* promoter) regulated by the *lac* repressor.

Vectors which are suitable for replication and expression in yeast contain a yeast replication start and a selective genetic marker for yeast. One group of such vectors includes so-called *ars* sequences (autonomous replication sequences) as origin of replication. These vectors are retained extrachromosomally within the yeast cell after the transformation and are replicated autonomously. Furthermore, vectors which contain all or part of the 2 $\mu$  (2 mikron) plasmid DNA from *Saccharomyces cerevisiae* can be used. Such vectors will get integrated by recombination into 2 $\mu$  plasmids already existing within the cell, or replicate autonomously. 2 $\mu$  sequences are particularly suitable when high transformation frequency and high copy numbers are to be achieved.

Expression control sequences which are suitable for expression in yeast are, for example, those of highly expressed yeast genes. Thus, the promoters for the *TRP1* gene, the *ADHI* or *ADHII* gene, acid phosphatase (*PHO3* or *PHO5*) gene, isocytochrome gene or a promoter involved with the glycolytic pathway, such as the promoter of the enolase, glyceraldehyde-3-phosphate kinase (*PGK*), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase and glucokinases, can be used.

Vectors suitable for replication and expression in mammalian cells are preferably provided with promoting sequences derived from DNA of viral origin, e.g. from Simian virus 40 (SV40), Rous sarcoma virus (RSV),

adenovirus 2, bovin papilloma virus (BPV), papovavirus BK mutant (BKV), r mouse r human cytomegalovirus (CMV). Alternatively, the vectors may comprise promoters from mammalian expression products, such as actin, collagen, myosin etc., or the native promoter and control sequences which are normally associated with the desired gene sequence, i.e. the immunoglobulin H-chain or L-chain promoter.

5 Preferred vectors are suitable for both procaryotic and eucaryotic hosts and are based on viral replication systems. Particularly preferred are vectors comprising Simian virus promoters, e.g. pSVgpt or pSVneo, further comprising an enhancer, e.g. an enhancer normally associated with the immunoglobulin gene sequences, in particular the mouse Ig H- or L-chain enhancer.

10 The recombinant DNA coding for a recombinant antibody of the invention can be prepared, for example, by culturing a transformed host cell and optionally isolating the prepared DNA.

In particular, such DNA can be prepared by a method comprising

- a) preparing murine DNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity, e.g. by isolating the DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA using DNA probes, or by isolating mRNA from a suitable hybridoma cell line and preparing
- 15 cDNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity using oligonucleotide primers,
- b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule, e.g. by isolating the desired DNA(s) from a suitable source, e.g. from a genomic library or a cDNA library using DNA probes,
- 20 c) synthesizing DNA coding for the desired spacer group by chemical methods,
- d) constructing recombinant genes encoding the recombinant antibodies by incorporating the DNA of step a) and, optionally, b) and/or c) into appropriate hybrid vectors,
- e) transferring the obtained hybrid vectors into a recipient host cell or retrieving the DNA coding for the recombinant genes and transferring the unlinked DNA into a recipient host cell,
- 25 f) selecting and culturing the transformed host cell, and
- g) optionally isolating the desired DNA.

The DNA according to step a) of the process described above can be obtained by isolation of genomic DNA or by preparation of cDNA from isolated mRNA. Genomic DNA from hybridoma cells is isolated by methods known in the art which include steps for disruption of the cells, e.g. by lysis in presence of detergents like Triton™, extracting the DNA, e.g. by treatment with phenol and CHCl<sub>3</sub>/isoamyl alcohol, and precipitation of DNA.

30 The DNA is fragmented, conveniently by one or more restriction endonucleases, the resulting fragments are replicated on a suitable carrier, e.g. nitrocellulose membranes, and screened with a DNA probe for the presence of the DNA sequences coding for the polypeptide sequence of interest, in particular for the presence of the rearranged H- and L-chain Ig gene loci. By this procedure DNA fragments are found that contain inserts with heavy chain V, D and J regions and light chain V and J regions, respectively, together with a leader sequence and introns, if any. cDNA from hybridoma cells is likewise prepared by methods known in the art, e.g. by extracting total cellular RNA, isolating mRNA by a suitable chromatographic method, e.g. chromatography on oligo(dT)-cellulose, synthesizing cDNA with a mixture of deoxynucleotide triphosphates and reverse transcriptase in the presence of oligonucleotide primers complementary to suitable regions in the murine immunoglobulin heavy and light chain constant domain genes, and isolating the cDNA. As a tool simplifying DNA isolation,

40 the desired genomic DNA or cDNA may be amplified using polymerase chain reaction (PCR) technology. PCR involves repeated rounds of extension from two primers specific for DNA regions at each end of the gene. Preferably, cDNA transcripts of total mRNA from the suitable hybridoma cell line is treated in a heating/cooling cycle with Taq DNA polymerase in the presence of primers tailored to hybridize to Ig H- and L-chain variable domains, respectively.

45 Genomic DNA or cDNA according to step b) of the process described above is isolated from suitable bacterial or mammalian cells according to methods known in the art. Preferably, the methods as described under a) are used, substituting the corresponding source cells for the murine hybridoma cells and using DNA probes designed to hybridize with the desired signal sequences or the genes coding for the desired effector molecules.

50 In bacteria wherein separation of mRNA from total RNA is not possible with oligo(dT)-cellulose, cDNA is prepared from total RNA using corresponding oligonucleotide primers. The DNA isolation is simplified considerably by the PCR technology.

DNA according to step c) is prepared by conventional chemical and enzymatic methods, e.g. by chemical synthesis of oligonucleotides of between thirty and sixty bases with overlapping complementary sequences,

55 hybridization of such oligonucleotides, and enzymatic ligation, optionally after filling-in of missing bases with suitable enzymes in the presence of the corresponding deoxynucleotide triphosphates.

The DNA probe for the mouse variable chain domains may be a synthetic DNA, a cDNA derived from mRNA coding for the desired immunoglobulin or a genomic DNA or DNA fragment of known nucleotide sequence. As

probes for the detection and/or amplification of the rearranged loci of the variable domains of L-/H-chains, DNA fragments of known nucleotide sequences of adjacent conserved variable or constant domains are selected which constitute the Ig loci of the L-/H-chain in the mammal from which the DNA is derived, e.g. Balb/c mice. The DNA probe is synthesized by chemical methods or isolated from suitable tissue of an appropriate mammal, e.g. Balb/c mouse liver, and purified by standard methods. If required, the probe DNA is labelled, e.g. radioactively labelled by the well-known nick-translation technique, then hybridized with the DNA library in buffer and salt solutions containing adjuncts, e.g. calcium chelators, viscosity regulating compounds, proteins, non-specific DNA and the like, at temperatures favoring selective hybridization.

Once a fragment has been identified which contains the desired DNA sequence, this fragment may be further manipulated to remove nonessential DNA, modified at one or both termini, and treated to remove all or a portion of intervening sequences, or the like.

The joining of the various DNA fragments in order to produce recombinant genes encoding the recombinant antibodies is performed in accordance with conventional techniques, for example, by blunt- or staggered-end ligation, restriction enzyme digestion to provide for appropriate cohesive termini, filling-in cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

The transfer of the recombinant DNAs, e.g. the transfer of hybrid vectors, and the selection of transformed cells is described below.

Moreover, the invention relates to host cells transformed with the recombinant DNAs described above, namely host cells which are transformed with a DNA encoding the heavy chain and/or a DNA encoding the light chain of the desired recombinant antibody, in particular host cells transformed with a DNA encoding the preferred single-chain recombinant antibody.

More specifically, the invention concerns a host cell which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter and a DNA coding for a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer which DNA is controlled by said promoter.

Furthermore, the invention pertains to a host cell which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

In particular, the present invention relates to a process for the production of a protein of the invention selected from the group consisting of a heavy chain murine variable domain, a light chain murine variable domain, a heavy chain murine variable domain and a light chain murine variable domain, a single-chain recombinant antibody, a fusion protein, and a fusion protein further comprising a peptide facilitating purification, a cleavage site and a peptide spacer comprising culturing a host, e.g. *E. coli*, which has been transformed with a hybrid vector comprising an expression cassette comprising a promoter operably linked to a first DNA sequence encoding a signal peptide linked in the proper reading frame to a second DNA sequence encoding said protein, and isolating said protein.

The host cells of the present invention have to be capable of culture *in vitro*. Suitable host cells are of procaryotic or of eucaryotic origin and are, for example, bacterial cells, e.g. *E. coli*, yeasts, e.g. *Saccharomyces cerevisiae*, or mammalian cells. For the preparation of functional chimeric human/mouse antibodies the host cells have to be of higher eucaryotic origin to provide a suitable environment for the production of active antibodies, since the biosynthesis of functional tetrameric antibody molecules requires correct nascent polypeptide chain folding, glycosylation, and assembly.

Examples of suitable hosts are microorganisms which are devoid of or poor in restriction enzymes or modification enzymes, such as bacteria, in particular strains of *Escherichia coli*, for example *E. coli* X1776, *E. coli* Y1090, *E. coli* HB 101, *E. coli* W3110, *E. coli* HB101/LM1035, *E. coli* JA 221, *E. coli* DH5 $\alpha$ , *E. coli* K12, or *E. coli* CC118 strain, *Bacillus subtilis*, *Bacillus stearothermophilus*, *Pseudomonas*, *Haemophilus*, *Streptococcus* and others, and yeasts, for example *Saccharomyces cerevisiae* such as *S. cerevisiae* GRF 18. Further suitable host cells are cells of higher organisms, in particular established continuous human or animal cell lines, e.g. human embryonic lung fibroblasts L132, human malignant melanoma Bowes cells, HeLa cells, SV40 virus transformed kidney cells of African green monkey COS-7 or Chinese hamster ovary (CHO) cells, or cells of lymphoid origin, such as lymphoma, myeloma, hybridoma, trioma or quadroma cells, for example PA1, Sp2/0 or X63-Ag8.653 cells.

The above mentioned strains of *E. coli*, in particular *E. coli* CC118, are preferred as hosts.

The invention also concerns processes for the preparation of transformed host cells wherein suitable recipient host cells as described hereinbefore are transformed with a hybrid vector according to the invention, and the transformed cells are selected.

5 Transformation of microorganisms is carried out as described in the literature, for example for *S. cerevisiae* (A. Hinnen et al., Proc. Natl. Acad. Sci. USA 75: 1929, 1978), for *B. subtilis* (Anagnostopoulos et al., J. Bacteriol. 81: 741, 1981), and for *E. coli* (M. Mandel et al., J. Mol. Biol. 53: 159, 1970).

Accordingly, the transformation procedure of *E. coli* cells includes, for example,  $\text{Ca}^{2+}$  pretreatment of the cells so as to allow DNA uptake, and incubation with the hybrid vector. The subsequent selection of the transformed cells can be achieved, for example, by transferring the cells to a selective growth medium which allows separation of the transformed cells from the parent cells dependent on the nature of the marker sequence of the vector DNA. Preferably, a growth medium is used which does not allow growth of cells which do not contain the vector. The transformation of yeast comprises, for example, steps of enzymatic removal of the yeast cell wall by means of glucosidases, treatment of the obtained spheroplasts with the vector in the presence of polyethylene glycol and  $\text{Ca}^{2+}$  ions, and regeneration of the cell wall by embedding the spheroplasts into agar. Preferably, the regeneration agar is prepared in a way to allow regeneration and selection of the transformed cells as described above at the same time.

Transformation of cells of higher eucaryotic origin, such as mammalian cell lines, is preferably achieved by transfection. Transfection is carried out by conventional techniques, such as calcium phosphate precipitation, microinjection, protoplast fusion, electroporation, i.e. introduction of DNA by a short electrical pulse which transiently increases the permeability of the cell membrane, or in the presence of helper compounds such as diethylaminoethyl dextran, dimethyl sulfoxide, glycerol or polyethylene glycol, and the like. After the transfection procedure, transfected cells are identified and selected, for example, by cultivation in a selective medium chosen depending on the nature of the selection marker, for example standard culture media such as Dulbecco's modified Eagle medium (DMEM), minimum essential medium, RPMI 1640 medium and the like, containing e.g. the corresponding antibiotic.

The host cells are transformed with the recombinant L-chain gene construct alone, with the recombinant H-chain gene construct alone, with both, either sequentially or simultaneously, or by using a vector construct comprising both the L-chain and H-chain genes, for example a recombinant single-chain antibody gene construct as indicated hereinbefore.

Preferred are host cells transformed with a recombinant single-chain antibody gene construct comprising DNA coding for the heavy chain variable domain of an anti-c-erbB-2 antibody, DNA coding for a spacer group, DNA coding for the light chain variable domain of an anti-c-erbB-2 antibody and DNA coding for an effector molecule, in particular transfected with the preferred recombinant single-chain antibody gene construct as indicated hereinbefore. Further examples of host cells of the invention are cells transfected with similar recombinant plasmids which contain alternative orientations of the H- and L-chain gene constructs, and those incorporating additional DNA elements to facilitate high levels of expression of the recombinant antibodies.

The host cells of the invention are genetically stable, secrete recombinant antibodies of the invention of constant specificity and can be activated from deep-frozen cultures by thawing and recloning.

40 The transformed host cells are cultured by methods known in the art in a liquid medium containing assimilable sources of carbon, e.g. carbohydrates such as glucose or lactose, nitrogen, e.g. amino acids, peptides, proteins or their degradation products such as peptones, ammonium salts or the like, and inorganic salts, e.g. sulfates, phosphates and/or carbonates of sodium, potassium, magnesium and calcium. The medium furthermore contains, for example, growth-promoting substances, such as trace elements, for example iron, zinc, manganese and the like.

The medium is preferably so chosen as to exert a selection pressure and prevent the growth of cells which have not been transformed or have lost the hybrid vector. Thus, for example, an antibiotic is added to the medium if the hybrid vector contains an antibiotic resistance gene as marker. If, for instance, a host cell is used which is auxotrophic in an essential amino acid whereas the hybrid vector contains a gene coding for an enzyme which complements the host defect, a minimal medium deficient of said amino acid is used to culture the transformed cells.

Cells of higher eucaryotic origin such as mammalian cells are grown under tissue culture conditions using commercially available media, for example Dulbecco's modified Eagle medium (DMEM), minimum essential medium, RPMI 1640 medium and the like as mentioned above, optionally supplemented with growth-promoting substances and/or mammalian sera. Techniques for cell cultivation under tissue culture condition are well known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads, porous glass beads, ceramic cartridges, or other microcarriers.

Culturing is effected by processes which are known in the art. The culturing conditions, such as temperature, pH value of the medium and fermentation time, are chosen so that a maximum titer of the polypeptide or derivative of the invention is obtained. Thus, an *E. coli* or yeast strain is preferably cultured under aerobic conditions by submerged culture with shaking or stirring at a temperature of about 20°C to 40°C, preferably at about 30°C, and a pH value of 4 to 8, preferably of about pH 7, for about 4 to 30 hours, preferably until maximum yields of the polypeptide or derivative of the invention are reached.

When the cell density has reached a sufficient value, the culture is interrupted and the polypeptide or derivative can be isolated. If the hybrid vector contains a suitable secretion signal sequence, the polypeptide or derivative is secreted by the transformed cell directly into the culture medium. Otherwise, the cells have to be destroyed, for example by treatment with a detergent such as SDS, NP-40™, Triton™ or deoxycholic acid, lysed with lysozyme or a similarly acting enzyme, or disrupted by an osmotic shock or ultra-sound. Break-up of the cells will also be required if the signal sequence directs the secretion of the desired protein into the cell periplasm. If yeast is used as a host microorganism, the cell wall may be removed by enzymatic digestion with a glucosidase. Alternatively or additionally, mechanical forces, such as shearing forces (e.g. French press, Dyno mill and the like) or shaking with glass beads or aluminium oxide, or alternating freezing, for example in liquid nitrogen, and thawing, for example at 30°C to 40°C, as well as ultra-sound can be used to break the cells.

The cell supernatant or the solution obtained after centrifugation of the mixture obtained after breaking the cells, which contains proteins, nucleic acids and other cell constituents, is enriched in proteins, including the polypeptides of the invention, in a manner which is known *per se*. Thus, for example, most of the non-protein constituents are removed by polyethyleneimine treatment and the proteins including the polypeptides and derivatives of the invention are precipitated, for example, by saturation of the solution with ammonium sulfate or with other salts. Otherwise, the cell supernatant or lysate is directly pre-purified by filtering through suitable membranes and/or with chromatographic methods, for example affinity chromatography.

The recombinant antibodies and the monoclonal antibodies according to the invention can be used for the qualitative and quantitative determination of the extracellular domain of the growth factor receptor c-erbB-2. This is especially useful for the monitoring of tumor progression, for the decision whether a tumor is amenable to treatment with the recombinant or monoclonal antibodies of the invention, and for monitoring the treatment of tumor with chemotherapy. Tumors considered are those over-expressing c-erbB-2, for example breast and ovarian tumors.

In general, the monoclonal and the recombinant antibodies according to the invention can be used in any of the known immunoassays which rely on the binding interaction between the antibodies and the antigen, i.e. the extracellular domain of the c-erbB-2 protein. Examples of such assays are radio-, enzyme, fluorescence, chemiluminescence, immunoprecipitation, latex agglutination, and hemagglutination immunoassays, and, in particular, immunostaining methods.

The antibodies according to the invention can be used as such or in the form of enzyme-conjugated derivatives in an enzyme immunoassay. Any of the known modifications of an enzyme immunoassay can be used, for example soluble phase (homogeneous) enzyme immunoassay, solid phase (heterogeneous) enzyme immunoassay, single enzyme immunoassay or double (sandwich) enzyme immunoassay with direct or indirect (competitive) determination of the c-erbB-2 protein.

An example of such an enzyme immunoassay is a sandwich enzyme immunoassay in which a suitable carrier, for example the plastic surface of a microtiter plate or of a test tube, e.g. of polystyrene, polypropylene or polyvinylchloride, glass or plastic beads, filter paper, dextran etc. cellulose acetate or nitrocellulose sheets, magnetic particles or the like, is coated with a monoclonal antibody of the invention by simple adsorption or optionally after activation of the carrier, for example with glutaraldehyde or cyanogen bromide. Then test solutions containing the soluble c-erbB-2 protein and finally single-chain recombinant antibodies of the invention comprising a detectable enzyme, e.g. alkaline phosphatase, are added. The amount of the soluble c-erbB-2 protein in the test solution is directly proportional to the amount of bound recombinant antibody and is determined by adding an enzyme substrate solution. The enzyme substrate reaction results, for example, in a color change which can be observed by eye or with optical measuring devices.

The antibodies according to the invention can be used as such or in the form of radioactively labelled derivatives in a radioimmunoassay (RIA). As described above for enzyme immunoassays, any of the known modifications of a radioimmunoassay can be used.

The tests are carried out in an analogous manner to the enzyme immunoassays described above using a radioactive label, e.g. <sup>125</sup>I, instead of an enzyme label. The amount of immune complex formed which corresponds to the amount of c-erbB-2 protein present in the test solutions is determined by measuring the radioactivity of the immune complex.

For immunostaining cryosections of cryopreserved biopsy material or paraffin embedded tissue sections are treated with a solution containing a recombinant antibody of the invention comprising a detectable enzyme.

Bound recombinant antibody is detected by treatment with a suitable enzyme substrate, preferably an enzyme substrate which leads to a solid deposit (stain) at the site of the recombinant antibody of the invention. In place of recombinant antibodies comprising an enzyme, a recombinant antibody comprising streptavidin and a solution of a biotin-enzyme-conjugate may be used, which leads to high enzyme concentration at the site of the antibody and hence increased sensitivity of the immunostaining method. The solid deposit of the enzyme substrate is detected by inspection with a microscope, for example with a fluorescence microscope, or by scanning the optical density at the wavelength of the stain.

The use according to the invention of recombinant and/or monoclonal antibodies as described hereinbefore for the determination of c-erbB-2 protein also includes other immunoassays known *per se*, for example immunofluorescence assays, latex agglutination with antibody-coated or antigen coated latex particles, hemagglutination with antibody-coated or antigen-coated red blood corpuscles, evanescent light assays using an antibody-coated optical fibre and other direct-acting immunosensors which convert the binding event into an electrical or optical signal, or the like.

The invention also concerns test kits for the qualitative and quantitative determination of c-erbB-2 protein comprising recombinant antibodies of the invention and/or monoclonal antibodies of the invention and, optionally, adjuncts.

Test kits according to the invention for an enzyme immunoassay contain, for example, a suitable carrier, optionally freeze-dried solutions of a monoclonal antibody, optionally freeze-dried or concentrated solutions of a recombinant antibody comprising an enzyme or streptavidin, solutions of an enzyme-biotin conjugate if a recombinant antibody comprising streptavidin is used, enzyme substrate in solid or dissolved form, standard solutions of c-erbB-2 protein, buffer solutions, and, optionally, polypeptides or detergents for preventing non-specific adsorption and aggregate formation, pipettes, reaction vessels, calibration curves, instruction manuals and the like.

Test kits according to the invention for immunostaining contain, for example, optionally freeze-dried or concentrated solutions of a recombinant antibody comprising an enzyme or streptavidin, solutions of an enzyme-biotin conjugate if a recombinant antibody comprising streptavidin is used, enzyme substrate in solid or dissolved form, buffer solutions, and, optionally, pipettes, reaction vessels, calibration curves, instruction manuals and the like.

The recombinant and monoclonal antibodies of the invention can be used for the qualitative and quantitative determination of c-erbB-2 protein. Due to the fact that the growth factor receptor c-erbB-2 is overexpressed in certain tumor types, for example breast and ovarian tumors, the antibodies are particularly well suited for detection and monitoring of the mentioned tumors. In addition, radiolabelled derivatives of the antibodies of the invention may be used for the *in vivo* localization of tumors in a patient using radioscanning techniques. To that end, radiolabelled derivatives of antibodies of the invention are injected into the patient, and the patient scanned with a gamma imager at regular intervals. Cells over-expressing the growth factor receptor c-erbB-2 will take up more radioactive antibodies than other tissue and will be clearly recognized by the gamma imaging camera. Preferentially recombinant or monoclonal antibodies labelled with <sup>131</sup>I or with <sup>99m</sup>Tc are used for radioscanning in amounts of 3 to 8 µg representing 15 to 30 µCi per kg body weight.

The antibodies of the invention can further be used for the isolation and purification of the c-erbB-2 protein from natural sources or from transformed host cells by immunoaffinity chromatography.

Furthermore, the monoclonal antibodies and the recombinant antibodies of the invention, in particular recombinant antibodies comprising an effector molecule, especially a toxin, in particular *Pseudomonas* exotoxin, are useful for the treatment of patients with tumors over-expressing the growth factor receptor c-erbB-2, for example breast or ovarian tumors. If it is desired, tumor therapy may comprise applying more than one, e.g. two different, antibodies of the invention, for example applying both FRP5 and FWP51. The recombinant antibodies comprising a phosphatase may be used in connection with a phosphorylated prodrug such as mitomycin phosphate or etoposide phosphate, thus enabling the conversion of the active drug to the prodrug at the site of the tumor.

The invention therefore also concerns pharmaceutical compositions for treating tumors over-expressing the growth factor receptor c-erbB-2 comprising a therapeutically effective amount of a recombinant antibody or of a monoclonal antibody according to the invention and a pharmaceutically acceptable carrier. Preferred are pharmaceutical compositions for parenteral application. Compositions for intramuscular, subcutaneous or intravenous application are e.g. isotonic aqueous solutions or suspensions, optionally prepared shortly before use from lyophilized or concentrated preparations. Suspensions in oil contain as oily component the vegetable, synthetic or semi-synthetic oils customary for injection purposes. The pharmaceutical compositions may be sterilized and contain adjuncts, e.g. for conserving, stabilizing, wetting, emulsifying or solubilizing the ingredients, salts for the regulation of the osmotic pressure, buffer and/or compounds regulating the viscosity, e.g. sodium carboxymethylcellulose, carboxymethylcellulose, sodium carboxymethylcellulose, dextran, polyvinylpyrrol-

dine or gelatine.

The pharmaceutical compositions of the invention contain from approximately 0.01% to approximately 50% of active ingredients. They may be in dosage unit form, such as ready-to-use ampoules or vials, or also in lyophilized solid form.

In general, the therapeutically effective dose for mammals is between approximately 5 and 25 µg of a recombinant antibody of the invention or of a monoclonal antibody of the invention per kg body weight depending on the type of antibody, the status of the patient and the mode of application. The specific mode of administration and the appropriate dosage will be selected by the attending physician taking into account the particulars of the patient, the state of the disease, the type of tumor treated, and the like. The pharmaceutical compositions of the invention are prepared by methods known in the art, e.g. by conventional mixing, dissolving, confectioning or lyophilizing processes. Pharmaceutical compositions for injection are processed, filled into ampoules or vials, and sealed under aseptic conditions according to methods known in the art.

The invention particularly concerns the monoclonal antibodies, the hybridoma cell lines, the recombinant single-chain antibodies, the recombinant DNAs, the transformed host cells, and the methods for the preparation thereof as described in the Examples. The following examples illustrate the invention but do not limit it to any extent.

#### Abbreviations

20	ATP	adenosine triphosphate
	BSS	Earle's balanced salt solution
	BSA	bovine serum albumin
	DEAE	diethylaminoethyl
	DMEM	Dulbecco's modified Eagle's medium
25	dNTP	deoxynucleotide triphosphate
	DTT	dithiothreitol
	EDTA	disodium ethylenediaminetetraacetate
	EGF	epidermal growth factor
	EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
30	FCS	fetal calf serum
	HAT medium	hypoxanthine, aminopterin and thymidine medium
	HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
	HT medium	hypoxanthine and thymidine medium
	Ig	immunoglobulin
35	IPTG	isopropyl-β-thiogalactoside
	MAb	monoclonal antibody
	PBS	phosphate-buffered saline
	PCR	polymerase chain reaction
	PMSF	phenylmethylsulfonyl fluoride
40	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	Tris	Tris-(hydroxymethyl)-aminomethane
	U	unit
	V <sub>L</sub>	light chain variable domain
	V <sub>H</sub>	heavy chain variable domain
45	XP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt

#### Examples

##### Example 1. Preparation of hybridoma cell lines FRP5, FSP16, FWP51 and FSP77

50 1.1 Source of antigen and immunization of Balb/c mice: The SKBR3 human breast tumor cell line (ATCC HTB 30), isolated in 1970 from a pleural effusion of a breast cancer patient, expresses approximately  $1 \times 10^6$  molecules of the c-erbB-2 receptor protein per cell.  $20 \times 10^6$  SKBR3 cells in PBS are injected subcutaneously and/or intra-peritoneally into Balb/c mice. The cells are mixed 1:1 (v/v) with complete Freund's adjuvant. The injections are repeated a total of five times over the period of approximately 3 months replacing Freund's incomplete adjuvant for complete adjuvant. The final injection of cells is given three days before the fusion.

55 1.2 Cell fusion: Immunized mice are sacrificed and their splenocytes fused according to conventional methods (Koehler & Milstein, Nature 256:495, 1976). Spleen cells are mixed at a 5:1 to 10:1 ratio with the fusion



partner, the mouse myeloma cell line PA1 (Stok et al., Research Disclosure #21713, 1982), in the presence of 41 % polyethylene glycol 4000 (Merck). Fused cells are plated at a density of  $1 \times 10^6$  cells per well in 24-well microtiter plates in peritoneal macrophages and fed 3 times per week with standard HAT selection medium for 2 weeks followed by 2 weeks of HT medium. When the growth of hybridoma cells becomes visible, the supernatants are screened as described in Example 1.3. Positive hybridomas are cloned and stored.

**1.3 Antibody detection in hybridoma supernatants:** Culture fluids of growing hybridomas are tested for the presence of anti-c-erbB-2 antibody using a protocol involving two steps, immunofluorescence and immunoprecipitation.

**1.3.1 Immunofluorescence:** In the first step, hybridoma supernatants are tested for their immunofluorescent staining of mouse cells expressing high levels of the human c-erbB-2 protein. To isolate these cells the HC11 mouse mammary epithelial cell line (Ball et al., EMBO J. 7: 2089, 1988) is transfected according to conventional, previously described methods (Graham & van der Eb, Virology 52: 456, 1973) with a plasmid expressing the human c-erbB-2 protein (Masuko et al., Jpn. Cancer Res. 80: 10, 1989) and with the plasmid pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1: 327, 1982) which encodes the gene for resistance to the drug G418. Transfected cells are selected 2 weeks in medium containing 200 µg/ml G418 (Geneticin, Gibco-BRL). Individual clones are selected and analyzed for expression of the human c-erbB-2 protein using conventional protein blotting techniques (Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350, 1979). A clone expressing high levels of the human c-erbB-2 protein (clone R1#11) is selected and used in the immunofluorescent assay. Non-transfected HC11 cells serve as control cells.

The assay is done in the following manner: The cells (R1 #11 or HC11) are grown in RPMI medium containing 8 % heat inactivated FCS (Amimed), 10 ng/ml EGF (Inotech) and 5 µg/ml insulin (Sigma) for 1-2 days on fibronectin (Boehringer Mannheim) coated cover slips. Fibronectin coated cover slips are prepared and stored at room temperature and they are used routinely for screening. The coverslips are rinsed in PBS containing calcium and magnesium and fixed by treatment for 10 min with 3.7 % formaldehyde (v/v in PBS). To reduce the non-specific binding the coverslips are incubated 20 min in PBS containing 3 % BSA (Sigma). The coverslips are washed in PBS and in water, then allowed to dry at room temperature. 20 - 30 µl of hybridoma supernatants are added to circled areas on a coverslip which is incubated 1 - 2 h at room temperature in a humidified atmosphere. The coverslips are then washed three times with PBS containing 0.05 % Triton-X100™ (Fluka) and incubated an additional hour with anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham). After three washes with PBS and one wash with water the cells are screened for fluorescence using a fluorescence microscope and a water immersion lens. Those hybridoma supernatants which are positive are screened in the second step described in Example 1.3.2.

**1.3.2 Immunoprecipitation and protein blotting analysis:** The SKBR3 human breast tumor cells express approximately  $1 \times 10^6$  molecules of the c-erbB-2 protein per cell. A cell lysate is prepared by extracting approximately  $4 \times 10^6$  cells in 1 ml of buffer containing 1 % Triton-X100™ (Fluka), 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.15 M NaCl, 1 mM PMSF (Boehringer Mannheim), 80 µg/ml aprotinin (Boehringer Mannheim), 50 µg/ml leupeptin (Boehringer Mannheim), and 4 µg/ml pepstatin (Boehringer Mannheim). 200 - 500 µl supernatant of hybridomas which are positive in the immunofluorescence assay described in Example 1.3.1 are incubated with 100 µl of the SKBR3 extract (2.5 - 4.0 mg/ml). This amount of extract contains approximately 50 - 100 ng of c-erbB-2 protein. The hybridoma supernatants and SKBR3 extract are incubated overnight on ice, then 1 µl of the IgG fraction of sheep anti-mouse Ig (ICN Immunobiologicals) is added. The complexes are collected by the addition of Protein-A Sepharose™ (Pharmacia), washed with TNET (140 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 % Triton X-100™) and water, boiled in sample buffer (80 mM Tris-HCl, pH 6.8, 0.2 % SDS, 10 % glycerol) and the supernatants loaded onto 8 % SDS-PAGE. The proteins are electrophoresed and blotted onto PVDF membranes (Millipore) using a technique originally described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350, 1979) with some modifications. The proteins are transferred using a semi-dry blotter (G. Frobel, Model 1004.01) following the instructions of the manufacturer. The membranes are blocked in PBS containing 0.5 % gelatin (Merck) for 1 h at 37°C. The membranes are washed twice for 5 min at 37°C in PTG (PBS containing 0.02 % gelatin (Merck) and 0.25 % Triton-X100™ (Fluka)). The c-erbB-2 protein is detected by incubating the membrane 45 min at 37°C in PTG containing an antiserum which is raised against the carboxy terminal 13 amino acids of the c-erbB-2 protein (Gullick et al., Int. J. Cancer 40: 246, 1987, antiserum 21N). The membranes are washed 3 times for 5 min at 37°C in PTG. The membrane-bound 21N antiserum is detected by incubating the membrane in PTG containing 0.1 µCi/ml <sup>125</sup>I-labeled protein-A (Amersham). The membranes are washed 4 times for 5 min at 37°C in PTG and exposed to X-ray film. The hybridomas whose supernatants are able to specifically immunoprecipitate the c-erbB-2 protein are grown for single cell cloning and further characterization described below.

## Exempl 2. Characterization of c-erbB-2 specific MABs

2.1 Hybridoma storage and processing: Hybridoma FRP5, FSP16, FWP51 and FSP77 secreting anti-c-erbB-2 MAB FRP5, FSP16, FWP51 and FSP77, respectively, can be grown in culture, frozen at -80°C or in liquid nitrogen and recultivated. The cells are cloned by the method of limiting dilution and have been deposited with the European Collection of Animal Cell Lines in England. The hybridoma cell lines have the following access numbers: FRP5: 90112115, FSP16: 90112116, FSP77: 90112117, FWP51: 90112118. The cells are expanded by forming ascites in Balb/c mice primed with pristane. The antibodies are purified from the ascites by ammonium sulfate precipitation and ion exchange chromatography on DE 52 DEAE-cellulose columns (Whatman). Purified MABs are stored in PBS at -80°C.

2.2 Isotyping of the MABs: The isotype of the MABs FRP5, FSP16, FWP51 and FSP77 is determined by ELISA analysis with rabbit antisera to mouse Ig classes and sub-classes (Biorad Mouse Typing TMSub Isotyping Kit™) as per manufacturer's suggested procedure. MABs FRP5, FWP51, and FSP77 are of the IgG1 isotype, while FSP16 is of the IgG2b isotype. The light chains of all the MABs are of the kappa type.

2.3 Flow cytometry: A FACS analysis using the c-erbB-2 specific MABs is carried out as follows: SKBR3 human breast tumor cells are trypsinized, washed in FACS medium (BSS containing 10 µM sodium azide, 4 % FCS and 25 mM EDTA), and  $1 \times 10^6$  cells are resuspended in 100 µl of FACS medium. Non-specific binding sites are blocked by incubating the cells 10 min at room temperature with 5 µl of goat serum. The SKBR3 cells are collected by centrifugation, resuspended in 50 µl of a 1:2 dilution of the supernatant made in FACS medium and incubated 45 min on ice. The cells are washed with 4 ml FACS medium, collected by centrifugation, resuspended in 50 µl of FACS medium containing a 1:20 dilution of anti-mouse Ig, fluorescein-linked whole antibody from sheep (Amersham), and incubated for 30 min on ice. 4 ml of FACS medium are added, the cells are collected by centrifugation, resuspended in 100 µl of FACS medium and analyzed without fixation for their fluorescence in a Becton-Dickinson FACScan™. As a control, SKBR3 cells are incubated with a non-reacting IgG1 MAB (1236S31-3). The FACS analysis shows that the SKBR3 cells treated with MAB FRP5, FSP16, FWP51, and FSP77 have a higher fluorescence than cells treated with the control MAB. These results show that the MABs bind to the extracellular domain of the c-erbB-2 protein.

2.4 Binding domain of c-erbB-2 specific MABs: MABs FRP5 and FSP77 are covalently linked with  $^{125}\text{I}$  (as carrier free sodium  $^{125}\text{I}$ iodide, Amersham) to a specific activity of 1 µCi/µg using Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril, Sigma) according to a standard protocol (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, p. 330). Competition experiments are conducted by incubating SKBR3 cells ( $0.5 - 1 \times 10^5$  cells per 15 mm well, Nunc™ 4-well multidish) with 250 µl RIA buffer (120 mM NaCl, 50 mM HEPES, pH 7.8, 1 mM EDTA, 2 % BSA) containing labeled FRP5 or FSP77 and varying amounts of unlabeled MAB FRP5, FSP16, FWP51 and FSP77 for 2 h at 4°C. The cells are washed 5 times with the RIA buffer, solubilized in 0.5 ml 1 % Triton X-100™, 10 % glycerol, 20 mM HEPES, pH 7.4, for 30 min at room temperature and the bound radioactivity is measured in a gamma counter. The results show that MABs FRP5 and FSP16 compete with each other for binding to SKBR3 cells which suggests that these 2 MABs bind to the same domain on the c-erbB-2 protein. MABs FWP51 and FSP77 neither compete with each other nor with FRP5 or FSP16 for binding to the c-erbB-2 protein. In conclusion, the panel of 4 MABs bind to 3 different domains of the extracellular portion of the c-erbB-2 membrane receptor tyrosine kinase.

## Example 3. Isolation of RNA from the hybridoma cell line FRP5

3.1 Growth of FRP5 cells: FRP5 hybridoma cells ( $1 \times 10^8$ ) are grown in suspension culture at 37°C in DMEM (Seromed) further containing 10 % FCS (Amimed), 1 mM sodium pyruvate (Seromed), 2 mM glutamine (Seromed), 50 µM 2-mercaptoethanol and 100 µg/ml of gentamycin (Seromed) in a humidified atmosphere of air and 7.5 % CO<sub>2</sub> in 175 cm tissue culture flasks (Falcon 3028). The cells are harvested by centrifugation, washed once in PBS, flash frozen in liquid nitrogen and kept frozen as a pellet at -80°C in a clean, sterile plastic capped tube.

3.2 Extraction of total cellular RNA from FRP5 cells: Total RNA is extracted using the acid guanidinium thiocyanate-phenol-chloroform method described by Chomczynski & Sacchi (Anal. Biochem. 162: 156, 1987). Cell pellets of FRP5 cells ( $1 \times 10^8$ ) are thawed directly in the tube in the presence of 10 ml of denaturing solution (4 M guanidinium thiocyanate (Fluka), 25 mM sodium citrate, pH 7.0, 0.5 % N-lauroylsarcosine (Sigma), 0.1 M 2-mercaptoethanol). The solution is homogenized at room temperature. Sequentially, 1 ml of 2 M sodium acetate, pH 4, 10 ml of phenol (water saturated) and 2 ml of chloroform-isoamyl alcohol mixture (49:1) are added to the homogenate. The final suspension is shaken vigorously for 10 sec and cooled on ice for 15 min. The samples are centrifuged at  $10,000 \times g$  for 20 min at 4°C. After centrifugation, RNA which is present in the aqueous phase is mixed with 10 ml of isopropanol and placed at -20°C for 1 h. The RNA precipitate is collected

by centrifugation, the pellet dissolved in 3 ml water and the RNA reprecipitated by addition of 1 volume of isopropanol at -20°C. After centrifugation and washing the pellet in ethanol, the final pellet of RNA is dissolved in water. The method yields approximately 300 µg of total cellular RNA. The final purified material is stored frozen at -20°C.

- 5     **3.3 Isolation of poly(A) containing RNA:** Poly(A) containing RNA is selected from total RNA by chromatography on oligo(dT)-cellulose (Boehringer Mannheim) as described originally by Edmonds et al. (Proc. Natl. Acad. Sci. USA 68: 1336, 1971) and modified by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982, p. 197). The poly(A)-containing RNA is prepared as described in the published procedure with the exception that the RNA is eluted from the oligo(dT)-cellulose with water rather than SDS-containing buffer. The poly(A)-containing RNA is precipitated with ethanol and collected by centrifugation. The yield of poly(A)-containing RNA is approximately 30 µg from 300 µg of total cellular RNA. The final purified material is stored frozen at -20°C.

**Example 4. Cloning of functional heavy and light chain rearrangements from the FRP5 hybridoma cell line**

- 15     Poly(A)-containing RNA isolated from FRP5 hybridoma cells as described in Example 3.3 provides the source for cDNA synthesis and subsequent amplification of V-region minigenes. Amplification products of the expected size are purified from agarose gels and cloned into appropriate vectors. Functional rearrangements are identified by sequencing.

20     **4.1 Oligonucleotides:**

MCK2 is designed to be complementary to a region in the murine immunoglobulin κ (kappa) constant minigene.

5'- TCACTGGATGGTGGGAAGATGGA - 3'

MCHC2 is designed to be complementary to a region in the murine immunoglobulin γ1 constant minigene.

25     5'-AGATCCAGGGGCCAGTGGATAGA-3'

The oligonucleotides VH1FOR, VH1BACK, VK1FOR, and VK1BACK are designed by Orlandi et al. (Proc. Natl. Acad. Sci. USA 86: 3833, 1989) to match consensus sequences.

VH1FOR : 5' - TGAGGAGACGGTGACCGTGGTGGTCCCTTGGCCCCAG - 3'

VH1BACK: 5' - AGGT(C/G)(C/A)A(G/A)CTGCAG(G/C)AGTC(T/A)GG - 3'

30     VK1FOR: 5' - GTTAGATCTCCAGCTTGGT(c/G)C(C/G) - 3'

VK1BACK: 5' - GACATTCAGCTGACCCAGTCTCCA - 3'

- 4.2 **cDNA synthesis:** 55 ng of poly(A)-containing RNA is dissolved in a buffer containing 50 mM Tris-HCl, pH 8.3, 3 mM magnesium chloride, 10 mM DTT, 75 mM KCl, 400 µM dNTPs (N = G, A, T and C), 100 µg BSA (molecular biology grade, Boehringer Mannheim), 100 U RNase inhibitor (Boehringer Mannheim), 25 pmol MCK2 and 25 pmol MCHC2. The RNA is denatured at 70°C for 5 min and then chilled on ice for 2 min. After addition of 200 U of MMLV reverse transcriptase (Gibco, BRL) cDNA synthesis is achieved by incubation for 1 h at 37°C.

- 4.3 **Polymerase chain reaction:** One tenth of the cDNA reaction is used for DNA amplification in buffer containing 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM β-mercaptoethanol, 200 µM dNTPs (N= G, A, T and C), 0.05 % Tween-20™ (Merck), 0.05 % NP-40™ (Merck), 10 % DMSO (Merck), 25 pmol oligonucleotide 1 (see below), 25 pmol oligonucleotide 2 (see below) and 2.5 U Amplitaq™ DNA polymerase (Perkin Elmer Cetus). Taq polymerase is added after initial denaturation at 93°C for 1 min and subsequent annealing at 37°C. In the first 4 cycles primer extension is performed at 71°C for 0.2 min, denaturation at 93°C for 0.01 min and annealing at 37°C for 0.2 min. For the last 25 cycles the annealing temperature is raised to 62°C. Finally, amplification is completed by a 3 min primer extension step at 71 °C.

	PCR Product	oligonucleotide 1	oligonucleotide 2
50	HC	MCHC2	VH1BACK
	H	VH1FOR	VH1BACK
	LC	MCK2	VK1BACK
55	L	VK1FOR	VK1BACK

4.4 **Modification and purification:** Amplified material is extracted with CHCl<sub>3</sub> and precipitated with ethanol in the presence of 200 mM LiCl. To facilitate cloning, blunt ends are created by a 3 min treatment with 1 U T4

DNA polymerase (Boehringer Mannheim) in 66 mM Tris-acetate, pH 7.9, 132 mM potassium acetate, 20 mM magnesium acetate, 1 mM DTT, 200 µg/ml BSA (molecular biology grade, Boehringer Mannheim), and 400 µM dNTPs (N = G, A, T and C). The polymerase is inactivated by heating for 15 min at 65°C before phosphorylation of the DNA with 10 U T4 polynucleotide kinase (Pharmacia) at 37°C for 1 h. For this purpose the buffer is adjusted to 50 mM EDTA and 1 mM ATP. The modified amplification products are separated on a 1.2 % (w/v) agarose gel (ultra pure DNA grade agarose, Biorad) and DNA of the expected size is eluted by means of DEAE NA 45 membranes (Schleicher & Schuell).

4.5 Ligation: Bluescript™ KS+ (70 ng) linearized with XbaI, treated with Klenow DNA polymerase (Boehringer Mannheim) to give blunt ends and dephosphorylated with calf intestinal phosphatase, and 30 ng of purified amplification product are ligated using 0.5 U T4 DNA ligase (New England Biolabs) in 50 mM Tris-HCl, pH 7.8, 10 mM magnesium chloride, 10 mM DTT, and 0.8 mM ATP overnight at 16°C. One half of the ligation mixture is used to transform *E. coli* K803 to obtain ampicillin resistant colonies. These are screened for the desired ligation products using a NaOH based plasmid "miniprep" method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The following plasmids are obtained:

	PCR product	Plasmid clones
	HC	pMZ15/1
		pMZ15/2
	H	pMZ16/1
		pMZ16/2
	L	pMZ17/1
		pMZ17/2
	LC	pMZ18/1
		pMZ18/2

4.6 Sequencing: Sequencing is done using Sequenase™ kits (United States Biochemicals) with T3 and T7 oligonucleotide primers according to procedures provided by the manufacturer.

Plasmid pMZ17/1 contains a non-functional rearrangement. Plasmid pMZ 17/2 contains an Ig-unrelated sequence. Plasmids pMZ18/1 (SEQ ID NO:2) and pMZ18/2 contain identical functional FRP5 kappa light chain variable domain inserts. Plasmids pMZ16/1 (SEQ ID NO: 1) and pMZ16/2 contain identical functional FRP5 heavy chain variable domain inserts. Plasmids pMZ15/1 and pMZ15/2 also contain FRP5 heavy chain variable domain inserts together with some constant region DNA. Plasmids pMZ16/1 and pMZ18/1 are used as a source for further subcloning steps.

#### Example 5. Construction of the MAb FRP5 single-chain Fv gene

5.1 Construction and sequence of a cloning linker for the heavy and light chain variable domain cDNAs: Using oligonucleotides, a linker sequence which allows the cloning of PCR amplified mouse heavy chain variable domain cDNA as a PstI/BstEII fragment and of PCR amplified mouse kappa light chain variable domain cDNA as a PvuII/BglII fragment is constructed. This creates an open reading frame in which heavy and light chain variable domains are connected by a sequence coding for the 15 amino acid stretch Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser. This amino acid linker has been shown to allow correct folding of an antigen binding domain present in heavy and light chain variable domains in a single-chain Fv (Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879, 1988).

For the construction of the cloning linker the 6 complementary oligonucleotides 1A, 1B, 2A, 2B, 3A, 3B are used.

1A: 5'-CAAGCTTCTCAGGTACAACCTGCAGGAGGTACCGTTTCCTCTGGGG-3'

1B: 5'-GAAACGGTGACCTCCTGCAGTTGTACCTGAGAAGTCTTGCATG-3'

2A: 5'-TGGCGGTTCTGGTGGCGGTGGCTCCGGCGGTGGCGGTTCTGAC-3'

2B: 5'-GCCACCGCCGAGCCACCGCCACCAGAACCGCCACCGCCAGAG-3'

3A: 5'-ATCCAGCTGGAGATCTAGCTGATCAAAGCT-3'

3B: 5'-CTAGAGCTTTGATCAGCTAGATCTCCAGCTGGATGGATGTCAGAACC-3'

40 pM of oligonucleotides 1B, 2A, 2B, 3A are phosphorylated at the 5' end using T4 polynucleotide kinase (Boehringer Mannheim) in four separate reactions in a total volume of 20 µl following the method described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Oligonucleotides 1A and 3B are not phosphorylated in order to avoid self ligation of the linker in the final ligation reaction.

After the kinase reaction, the enzyme is inactivated by incubation at 70°C for 30 min. In three separate reactions, each containing 40 pM of two oligonucleotides in a total volume of 40 µl, non-phosphorylated 1A and phosphorylated 1B, phosphorylated 2A and phosphorylated 2B, and phosphorylated 3A and non-phosphorylated 3B are mixed. Hybridization of the oligonucleotides in the three reactions is carried out by heating to 95°C for 5 min, incubation at 65°C for 5 min and slowly cooling to room temperature. 10 µl from each of the three reactions are mixed, 4 µl of 10 x ligation buffer (Boehringer) and 4 units of T4 DNA ligase (Boehringer) are added and the total volume is adjusted to 40 µl with sterile water. The annealed pairs of oligonucleotides are ligated into one linker sequence for 16 h at 14°C. The reaction mixture is extracted with an equal volume of phenol/chloroform (1:1) followed by re-extraction of the aqueous phase with an equal volume of chloroform/isoamylalcohol (24:1). The aqueous phase is collected, 0.1 volumes of 3 M sodium acetate pH 4.8 and 2 volumes of ethanol are added, and the DNA is precipitated at -70°C for 4 h and collected by centrifugation. The resulting linker sequence has a SphI and a XbaI adaptor end. It is ligated to SphI and XbaI digested pUC19 in a reaction containing 100 ng of ligated linker and 200 ng of SphI/XbaI digested pUC19. After transformation into *E. coli* XL1 Blue™ (Stratagene), plasmid DNA from 4 independent colonies is isolated by the alkaline lysis mini-preparations method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). The DNA sequence of the linker cloned in pUC19 is determined by sequencing double stranded DNA in both directions with Sequenase II (United States Biochemicals) and pUC universal and reverse primers (Boehringer) following the manufacturer's protocol. Three out of the four recombinant pUC 19 isolates sequenced contain the correct linker sequence. One of them is designated pWW 19 and used in the further experiments. The sequence is shown in SEQ ID NO:3.

**5.2 Preparation of a plasmid for the subcloning of variable domains:** The Fv cloning linker sequence is derived as a 144 bp HindIII/SacI fragment from pWW19 and inserted into HindIII/SacI digested Bluescript™ KS+ (ex PvuII) (Stratagene) which contains no PvuII restriction sites. The resulting plasmid, pWW15, allows cloning of heavy and light chain variable domains as PstI/BstEII and PvuII/BglII fragments, respectively.

**5.2.1 Subcloning of the FRP5 heavy chain variable domain:** Plasmid pMZ16/1 is digested with PstI and BstEII and the 338 bp heavy chain variable domain fragment of FRP5 is isolated. It is cloned into PstI/BstEII digested pWW19 yielding the plasmid pWW31.

**5.2.2 Mutation of the FRP5 light chain variable domain and assembly of the Fv fusion gene:** To facilitate subcloning of the FRP5 light chain variable domain into the Fv cloning linker, a PvuII restriction site and a BglII restriction site are introduced at the 5' and 3' ends, respectively, of the coding region. The FRP5 light chain variable domain coding region is isolated as a SacI/BamHI fragment from pMZ18/1. SacI and BamHI are restriction sites of the Bluescript™ polylinker present in pMZ18/1. The fragment contains the complete light chain variable domain fragment of 392 bp amplified by PCR using the oligonucleotide MCK2 (see above). This fragment is mutated and amplified by PCR using the oligonucleotides

V<sub>L</sub>5':5'-GACATTCAGCTGACCAG-3' and

V<sub>L</sub>3':5'-GCCCCGTTAGATCTCCAATTTTGTCCCCGAG-3'

for the introduction of a PvuII restriction site at the 5' end (VL5') and a BglII restriction site at the 3' end (VL3') of the kappa light chain variable domain DNA. 20 ng of the FRP5 variable light chain SacI/BamHI fragment are used as a template in a 100 µl reaction following the PCR conditions described in Example 4.3. The amplified and mutated fragment is isolated after PvuII/BglII digestion as a 309 bp fragment from a 1.5 % agarose gel and cloned into PvuII/BglII digested pWW15 generating plasmid pWW41. The FRP5 kappa light chain variable domain is isolated as a BstEII/XbaI fragment from pWW41 and inserted into BstEII/XbaI digested pWW31. Thus the FRP5 heavy chain variable domain in pWW31 and the FRP5 kappa light chain variable domain are fused to one open reading frame. Double stranded DNA of three independent clones is sequenced with Sequenase II™ kit (United Biochemicals) in both orientations using pUC universal and reverse primers (Boehringer) following the manufacturer's protocol. One of the plasmids carrying the FRP5 heavy chain variable domain fused to the mutated FRP5 light chain variable domain is selected and designated pWW52. The sequence of the HindIII/XbaI insert in plasmid pWW52 is shown in SEQ ID NO:4.

#### Example 6. Construction of a single-chain Fv-phosphatase fusion gene expression plasmid

The MAb FRP5 single-chain Fv gene is fused to the bacterial alkaline phosphatase. This chimeric gene encodes a bifunctional molecule which retains binding activity to the c-erbB-2 protein and has enzymatic activity.

**6.1 Mutation of the single-chain Fv(FRP5) gene:** To allow gene fusion between the single-chain Fv(FRP5) encoding gene from pWW52 and the alkaline phosphatase gene *phoA* the stop codon at sequence position 729 to 731 in pWW52 (see Example 5.2.3) is deleted as follows: Plasmid DNA of pWW52 is digested with BstEII and BglII and the linker sequence and FRP5 light chain variable domain encoding fragment is isolated. In

another digestion, pWW52 is cleaved with BstEII and BclI. Thus, the large fragment containing vector sequences and the FRP5 heavy chain variable domain encoding sequence is isolated. The BstEII/BglII V<sub>L</sub> fragment is now inserted into BstEII/BclI cleaved pWW52 containing V<sub>H</sub>. In the resulting plasmid, pWW53, the BglII/BclI junction is determined by sequencing double stranded DNA as described above.

Sequence of the BglII/BclI junction in pWW53 (position numbers correspond to position numbers of the HindIII/XbaI insert in plasmid pWW52, SEQ ID NO:4):

#### BglII/BclI

ACA AAA TTG GAG ATC AAA GCT CTA GA

714 - 728 | 738 - 748

**6.2 Mutation of the *E. coli* alkaline phosphatase gene *phoA*:** For the construction of the Fv(FRP5)-*phoA* fusion gene the *E. coli* alkaline phosphatase gene *phoA* is mutated to generate a XbaI cleavage site in the coding region of *phoA* near the N terminus of the mature protein and a SacI cleavage site in the 3' untranslated region of *phoA*. This step facilitates the cloning of the mutated fragment. A pBR322 derivative carrying the recombinant transposon TnPhoA (Manoil & Beckwith, Proc. Natl. Acad. Sci. USA 82: 8129, 1985) is linearized by BglII cleavage. 20 ng of the linearized template DNA is used for a 100 µl PCR reaction carried out as described previously using oligonucleotides PhoA5' and PhoA3' as primers 1 and 2.

PhoA5': 5'-CCCTCTAGAGCCTGTTCTGGAAC-3'

PhoA3': 5'-CCCGAGCTCTGCCATTAAG-3'

Following XbaI/SacI digestion of the PCR products, a 1419 bp fragment is isolated from a 1.5 % agarose gel and inserted into XbaI/SacI digested plasmid pUC19. Ligation is carried out as described above. Ligated DNA is transformed into *E. coli* XL1 Blue™ (Stratagene). Thus, the open reading frame of the mutated *phoA* gene is fused in frame to the lacZ open reading frame of pUC19. To show that the mutated *phoA* gene expresses functional alkaline phosphatase, recombinant clones are plated onto LB agar plates containing 100 µg/ml ampicillin, 0.5 mM IPTG (Sigma), and 40 µg/ml XP (Boehringer). Following induction of the lac promoter of pUC19, a lacZ-*phoA* fusion protein is expressed. The phosphatase activity of this fusion protein converts the indicator XP to a blue dye. One of the blue colonies is isolated and the presence of the introduced restriction sites is confirmed by digestion of miniprep DNA with XbaI and SacI. Partial 5' and 3' DNA sequences of the mutated *phoA* gene are obtained by sequencing double stranded DNA as described above. The DNA sequences are included in the assembly of the final Fv(FRP5)-*phoA* fusion gene sequence shown in SEQ ID NO:5. The isolated plasmid is designated pWW61 and used for further subcloning steps.

**6.3 Construction of a FRP5 Fv-*phoA* expression plasmid:** From plasmid pWW19 (see Example 5.1.2) the cloning linker sequence is isolated as a HindIII/EcoRI fragment and inserted into HindIII/EcoRI digested plasmid pINIII-ompA-Hind (Rentier-Delrue et al., Nucl. Acids Res. 16: 8726, 1988) leading to plasmid pWW16.

From pWW61 (see Example 6.2) the mutated *phoA* gene is isolated as a XbaI/SacI fragment and inserted into XbaI/SacI digested pWW53. The resulting plasmid, pWW615, carries the Fv(FRP5) gene fused in frame to the mutated alkaline phosphatase gene. The Fv(FRP5)-*phoA* gene is isolated as a HindIII/SacI fragment from pWW615 and inserted into HindIII/SacI digested plasmid pWW16. This leads to the production of the Fv(FRP5)-*phoA* expression plasmid pWW616 (see below). All ligations are carried out as described above. Recombinant plasmids are transformed into *E. coli* XL1 Blue™ (Stratagene). The constructs are confirmed by restriction enzyme analysis of plasmid DNA isolated by an alkaline mini preparation method (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

In this construct the Fv single-chain antibody of FRP5, genetically fused to the alkaline phosphatase *phoA*, can be expressed in *E. coli* following induction with IPTG. The recombinant protein carries the *E. coli* outer membrane protein A (ompA) signal sequence at the N terminus (encoded by the pINIII-ompA-Hind vector) to facilitate secretion of the protein into the periplasmic space of *E. coli* expressor cells.

The sequence of the Fv(FRP5)-*phoA* fusion gene in expression plasmid pWW616 is shown in SEQ ID NO:5. Part of the *phoA* sequence is assembled from Chang et al., Gene 44: 121, 1986.

#### Example 7. Expression of Fv(FRP5)-*phoA* in *E. coli*

Plasmid pWW616 is transformed into the *phoA* negative *E. coli* strain CC118 (Manoil & Beckwith, Proc. Natl. Acad. Sci. USA 82: 8129, 1985). A recombinant single colony is grown overnight in 50 ml LB medium containing 70 µg/ml ampicillin. The overnight culture is diluted 1:10 in 500 ml fresh LB medium containing 70 µg/ml ampicillin and grown at 37°C to an OD<sub>550</sub> of 0.1. IPTG is added to a final concentration of 2 mM and exp-

repression is induced for 1.5 h at 37°C. The cells are harvested at 4°C by centrifugation at 4000 rpm for 25 min in a Beckman GPKR centrifuge. The supernatant of CC118/pWW616 is set aside on ice for preparation of Fv(FRP5)-phoA, see Example 7.2.

7.1 Isolation of Fv(FRP5)-phoA from the periplasmic proteins of CC118/pWW616: The bacterial pellet is suspended in 10 ml TES buffer (0.2 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) and kept on ice for 10 min. After centrifugation at 4°C for 10 min at 5000 rpm in a Heraeus minifuge, the supernatant is discarded and the washed pellet is suspended in 15 ml ice-cold TES, diluted (1:4) with water. The cells are kept on ice for 30 min and recentrifuged as above. The supernatant containing periplasmic proteins is recentrifuged at 45,000 x g for 15 min in a Beckman TL100 ultracentrifuge. The periplasmic extract is concentrated in an Amersham ultrafiltration unit through a YM10 membrane to a final volume of 2 ml. Following fivefold dilutions with PBS and reconcentration through the YM10 membrane five times, the 1:4 diluted TES buffer of the periplasmic extract is exchanged with PBS.  $\text{NaN}_3$  and protease inhibitors are added to the periplasmic proteins (2 ml in PBS) to the final concentration of 0.02 %  $\text{NaN}_3$ , 0.1 mM PMSF, 2  $\mu\text{g/ml}$  aprotinin, 1  $\mu\text{g/ml}$  leupeptin, and 1  $\mu\text{g/ml}$  pepstatin. The periplasmic extract is stored at 4°C.

7.2 Isolation of Fv(FRP5)-phoA from the concentrated supernatant of *E. coli* CC118/pWW616 cultures: The supernatant (500ml) of the induced *E. coli* culture CC118/pWW616 is filtered through a 0.45  $\mu\text{m}$  membrane. The filtrate is concentrated in an Amicon ultrafiltration unit through a YM10 membrane to a final volume of 10 ml in PBS as described above.  $\text{NaN}_3$  and protease inhibitors are added to the concentrated supernatant to the final concentrations indicated above. The concentration of Fv(FRP5)-phoA in the extracts is determined by densitometry in comparison to BSA standards of coomassie stained 9 % SDS-PAGE gels.

#### Example 8. Activity of Fv(FRP5)-phoA

8.1 Detection of c-erbB-2 in SKBR3 breast tumor cells by immunostaining using Fv(FRP5)-phoA: The Fv domain of Fv(FRP5)-phoA enables the molecule to bind to the extracellular domain of the c-erbB-2 protein. Bound Fv(FRP5)-phoA can be visualized by staining procedures using color substrates for the detection of alkaline phosphatase activity.

8.1.1 Fixation of cells: SKBR3 human breast tumor cells carrying about  $1 \times 10^6$  c-erbB-2 receptors per cell are grown on fibronectin coated glass cover slips. The cells are washed twice with PBS and then fixed with PBS / 3.7 % formaldehyde at room temperature for 30 min. The fixed cells are washed three times with PBS at room temperature. Unspecific binding sites are blocked by incubating the cells for 1 h with PBS / 3 % BSA at 37°C in a humid incubator. The cells are then washed twice with PBS.

8.1.2 Pretreatment of Fv(FRP5)-phoA: Alkaline phosphatase phoA from *E. coli* must be dimerized to be enzymatically active. In the periplasm of *E. coli* natural phoA is dimerized, i.e. two molecules of phoA are held together by two  $\text{Zn}^{2+}$  ions. The Fv(FRP5)-phoA is also produced as a dimer in *E. coli*. To increase binding of Fv(FRP5)-phoA to the antigen, the dimers are monomerized by adding EGTA to the solution. This step removes  $\text{Zn}^{2+}$  from the solution. Monomerized phosphatase can be re-dimerized by the addition of  $\text{Zn}^{2+}$ . EGTA is added to a final concentration of 5 mM to 200  $\mu\text{l}$  of 40 x concentrated supernatant or periplasmic proteins from CC118/pWW616 (see above). The solution is incubated at 37°C for 1 h just before use in the immunoassay.

8.1.3 Staining of cells: After blocking with PBS / 3 % BSA (see above) fixed cells are incubated for 1 h with pretreated Fv(FRP5)-phoA at a concentration of 1  $\mu\text{g/ml}$  at 37°C in a humidified incubator. The cells are washed three times with PBS at room temperature. The staining solution consists of 300  $\mu\text{l}$  naphthol AS-MX<sup>TM</sup> phosphate (Sigma, 13 mg/ml in dimethyl formamide), 8 mg of levamisole (Sigma), and 10 mg of Fast Red TR<sup>TM</sup> salt (Sigma) added to 9.7 ml of 100 mM Tris-HCl, pH 8.2, 1 mM  $\text{ZnCl}_2$ . This mixture is prepared and filtered through a 0.45  $\mu\text{m}$  filter immediately before use.  $\text{ZnCl}_2$  is added to the staining solution to allow re-dimerization of bound Fv(FRP5)-phoA and thereby activating the alkaline phosphatase. Cells are incubated in the Fast Red<sup>TM</sup> staining solution for 15 min at room temperature. The phosphatase activity is blocked after staining by washing the cells twice with PBS and once with 1 M  $\text{KH}_2\text{PO}_4$ . Glass cover slips are mounted with gel mount (Biomed). The cells are examined under a fluorescence microscope using green light for excitation. Stained SKBR3 cells show intense red cell surface fluorescence.

8.2 Detection of c-erbB-2 protein over-expression in immunoblots using Fv(FRP5)-phoA: Proteins from total cell lysates of SKBR3 cells over-expressing c-erbB-2 protein are separated by SDS-PAGE and blotted onto PVDF membrane (Millipore). For preparation of extracts and immunoblotting technique see Example 1.3.2. Free binding sites of the membrane are blocked by incubation for 1 h at room temperature in a solution containing 10 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 0.05 % Tween 20<sup>TM</sup> (BioRad), and 3 % BSA. Pretreated Fv(FRP5)-phoA (see Example 7.2.) is diluted in blocking solution to a final concentration of 0.1  $\mu\text{g/ml}$ . The membrane is incubated in the Fv(FRP5)-phoA solution for 1 h at room temperature and then washed three times for 5 min at room temperature in 10 mM Tris-HCl, pH 7.5, 0.9 % NaCl, 0.05 % Tween 20<sup>TM</sup> and once in 10 mM Tris-HCl,

pH 7.5, 0.9 % NaCl. For detection of bound Fv(FRP5)-phoA the membrane is incubated for 20 min at 37°C in the Fast Red™ substrate solution described in Example 7.3 with out levamisole. The reaction is stopped by washing the membrane twice in water. Fv(FRP5)-phoA specifically detects the 185 kD c-erbB-2 protein.

#### 5 Example 9. Expression and isolation of Fv(FRP5)-phoA from *E. coli*

9.1 Preparation of periplasmic extract: Plasmid pWW616 is transformed into the phoA negative *E. coli* strain CC118 according to standard procedures (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). A single colony is picked and grown overnight in LB medium containing 70 µg/ml  
10 ampicillin. The overnight culture is diluted 1:10 in fresh LB medium containing ampicillin and grown at 37°C to an OD<sub>660</sub> of 0.1. At this point expression of the Fv(FRP5)-phoA gene is induced by the addition of IPTG to a final concentration of 2 mM, and the cells are grown for an additional 1.5 to 2 h. The cells are harvested by centrifugation and treated with a mild osmotic shock which releases the periplasmic proteins into the supernatant. The proteins are concentrated in an Amersham ultrafiltration unit through a YM10 membrane.

9.2 Preparation of an antigen affinity column: The c-erbB-2 protein is isolated from insect cells infected with a baculovirus vector expressing the c-erbB-2 extracellular domain by standard methods (V.A. Luckow & M.D. Summers, Biotechnology 6: 47-55, 1988). MAb FSP77 is coupled to CNBR-activated Sepharose 4B™ (Pharmacia) following the instructions of the manufacturer. The insect cell lysates are incubated with the coupled  
20 MAb FSP77 in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 0.5 % Triton X-100™, 150 mM NaCl for 2 h at 4°C on a shaking platform. The beads are packed into a column and washed with pre-elution buffer consisting of 10 mM phosphate, pH 6.8, and 100 mM NaCl to remove non-specifically bound proteins. The c-erbB-2 protein is recovered from the column by treatment with a low pH elution buffer containing 100 mM glycine, pH 3.0, and 100 mM NaCl. The fractions from the column are collected into phosphate buffer, pH 8.0, in order to raise the pH. The c-erbB-2 extracellular domain is detected by running a part of each fraction on 8  
25 % SDS-PAGE gel, blotting onto PVDF membrane (Millipore) and treating the filter with MAb FSP77 followed by sheep anti-mouse IgG. Bound IgG is detected by <sup>125</sup>I-Protein-A treatment. The fractions containing the extracellular domain are pooled and the protein is coupled to CNBR-activated Sepharose 4B™ (Pharmacia) following the instructions of the manufacturer.

9.3 Isolation of Fv(FRP5)-phoA by affinity chromatography: The sepharose coupled to c-erbB-2 protein (Example 9.2) is incubated for 2-4 h at 4°C on a rocking platform with the periplasmic extract isolated as described in Example 9.1. The beads are packed into a column and washed with pre-elution buffer as in Example 9.2. The Fv(FRP5)-phoA protein is recovered by elution with the low pH elution buffer of Example 9.2. The fractions are monitored for the presence of the Fv(FRP5)-phoA by testing for phoA enzymatic activity using a standard protocol.  
35

#### Example 10. Immunoassay for c-erbB-2 protein in tumors

10.1 Preparation of tumor sections: To determine the level of c-erbB-2 protein in tumors, tumor tissue is pretreated to give either frozen tumor sections or paraffin-embedded tumor sections. Tumor pieces are quick  
40 frozen, then cut with a cryostat, collected onto 1 % gelatin-coated glass slides, and fixed with 4 % paraformaldehyde. Following several washes with PBS, the tumor tissue sections are ready for staining. Alternatively, tumor pieces are placed in 4 % paraformaldehyde for fixation, embedded in paraffin, then sections cut and collected onto polylysine-coated glass cover slips. To prepare the sections for staining, they are heated overnight at 56°C, dewaxed in xylene, stepwise rehydrated by washing in 95 %, 70 % and 35 % ethanol and water, and washed in PBS.  
45

10.2 Pretreatment of Fv(FRP5)-phoA: Since the dimer of the Fv(FRP5)-phoA as obtained from the *E. coli* periplasm does not bind optimally to the c-erbB-2 antigen, it is first monomerized. This is accomplished by treating the solution of Fv(FRP5)-phoA for 1 h at 37°C with EGTA at a final concentration of 5 mM. This treatment chelates the Zn<sup>2+</sup> ions which are important for maintaining the dimeric structure of Fv(FRP5)-phoA.

10.3 Staining of the tumor sections: Non-specific staining of the tumor sections prepared according to Example 10.1 is blocked by incubating the sections in PBS containing 3 % BSA. The blocked sections are incubated for 1 - 2 h with pretreated Fv(FRP5)-phoA (Example 10.2) at a concentration of 1 µg/ml in a humidified chamber at room temperature. The sections are washed three times with PBS at room temperature. The bound Fv(FRP5)-phoA protein is detected using Fast Red™ as a substrate for the alkaline phosphatase. The staining  
55 solution consists of 300 µl naphthol AS-MX phosphate (Sigma, 13 mg/ml in dimethylformamide), 8 mg of levamisole (an inhibitor of endogenous alkaline phosphatase, Sigma), and 10 mg of Fast Red TR™ salt (Sigma) added to 9.7 ml of 100 mM Tris-HCl, pH 8.2, and 1 mM ZnCl<sub>2</sub>. This mixture is prepared and filtered through a 0.45 µm filter immediately before use. ZnCl<sub>2</sub> is added to the staining solution to allow re-dimerization of the bound



Fv(FRP5)-phoA protein and activation of the alkaline phosphatase. The tumor sections treated with Fv(FRP5)-phoA are incubated in the Fast Red™ staining solution for 15 min at room temperature. After staining the phosphatase activity is blocked by washing the cells twice with PBS and once with 1 M KH<sub>2</sub>PO<sub>4</sub>. The glass cover slips are mounted with a glass mount. The cells are examined under a fluorescence microscope using green light for excitation. Positively stained cells show an intense red cell surface fluorescence.

Alternatively, the tumor sections treated with the Fv(FRP5)-phoA protein may be stained with naphthol AS-BI phosphate (Sigma) and New Fuchsin™ (Sigma), or with 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and Nitro Blue Tetrazolium™ (Sigma). The stained sections can then be viewed with a regular light microscope.

#### Example 11. Cloning of functional heavy and light chain rearrangements from the FWP51 hybridoma cell line

Poly(A)-containing RNA isolated from FWP51 hybridoma cells as described in Example 3.3 provides the source for cDNA synthesis and subsequent amplification of V-region minigenes. cDNA synthesis and amplification of FWP51 heavy and light chain variable domain cDNA by polymerase chain reaction is carried out as described in Example 4. Amplification products of the expected size are purified from agarose gels and cloned into appropriate vectors. Functional rearrangements are identified by sequencing.

**11.1 Subcloning of FWP51 heavy and light chain variable domain cDNA:** Material amplified according to Example 4.3 is extracted with CHCl<sub>3</sub> and precipitated in the presence of 200 mM LiCl. To facilitate cloning, the FWP51 heavy chain variable domain cDNA is cleaved with restriction enzymes PstI and BstEII, the fragment purified by agarose gel electrophoresis, and ligated to PstI and BstEII digested pWW15 DNA. The FWP51 light chain variable domain cDNA is cleaved with restriction enzymes PvuII and BglII, the fragment is purified by agarose gel electrophoresis, and ligated to PvuII and BglII digested pWW15 DNA (cf. Example 5). Ligation, transformation, and screening for the desired ligation products are carried out as described in Example 4.5.

The following plasmids are obtained:

	PCR product	Plasmid clones
	H	pWW15-VH51-1 pWW15-VH51-2 pWW15-VH51-3
	L	pWW15-VL51-1 pWW15-VL51-2 pWW15-VL51-3

**11.2 Sequencing:** Sequencing is done as described in Example 4.6.

Plasmids pWW15-VH51-1 (SEQ ID NO:6), pWW15-VH51-2, pWW15-VH51-3 contain identical functional FWP51 heavy chain variable domain inserts. Plasmids pWW15-VL51-1 (SEQ ID NO:7), pWW15-VL51-2, pWW15-VL51-3 contain identical functional FWP51 kappa light chain variable domain inserts. Plasmids pWW15-VH51-1 and pWW15-VL51-1 are used as a source for further subcloning steps.

#### Example 12. Construction of the MAb FWP51 single chain gene

**12.1 Assembly of the Fv fusion gene:** Plasmid pWW15-VH51-1 is digested with PstI and BstEII and the 342 bp heavy chain variable domain fragment of FWP51 is isolated. It is cloned into PstI/BstEII digested pWW15-VL51-1 yielding the plasmid pWW15-Fv51 (SEQ ID NO: 8).

**12.2 Mutation of the single-chain Fv(FWP51) gene:** To allow gene fusion between the single-chain Fv(FWP51) encoding gene from pWW15-Fv51 and effector genes the stop codon at sequence position 729 to 731 in pWW15-Fv51 (SEQ ID NO:8) is deleted as follows (see also Example 6.1): plasmid DNA of pWW15-Fv51 is digested with BstEII and BglII and the linker sequence and FWP51 light chain variable domain encoding fragment is isolated. In another digestion, pWW15-Fv51 is cleaved with BstEII and BclI. Thus, the large fragment containing vector sequences and the FWP51 heavy chain variable domain encoding sequence is isolated. The

BstEII/BglII VL fragment is now inserted into BstEII/BclI cleaved pWW15-Fv51 containing V<sub>H</sub>. The resulting plasmid pWW15-Fv51-ORF is used as a source for the construction of Fv(FWP51)-effector fusion genes.

#### Example 13. Construction of single-chain Fv-exotoxin A fusion gene expression plasmids

The MAb FRP5 and MAb FWP51 single-chain Fv genes are fused to a truncated bacterial toxin, exotoxin A (ETA) from *Pseudomonas aeruginosa*. These chimeric genes encode recombinant immunotoxins which selectively inhibit protein synthesis in c-erbB-2 expressing cells.

**13.1 Mutation of the Exotoxin A gene of *Pseudomonas aeruginosa* PAK:** For the construction of Fv-exotoxin A (Fv-ETA) fusion genes the ETA gene from *Pseudomonas aeruginosa* PAK is mutated to delete the original cell binding domain I at the N-terminus of the toxin and to generate a XbaI cleavage site at the former domain I/domain II boundary of the ETA coding region. Plasmid pMS150A (Lory et al., J. Bacteriol. 170: 714, 1988) is linearized by EcoRI cleavage. 20 ng of the linearized template DNA is used for a 100 µl PCR reaction carried out as described previously using the following oligonucleotides as primers 1 and 2.

1: 5' -CACGGAAGCTTAAGGAGATCTGCATGCTTCTAGAGGGCGGCA-  
GCCTGGCCGCGCTG-3'

2: 5' -GCGGATCGCTTCGCCCAGGT-3'

Following HindIII/SalI digestion of the PCR products, a 201 bp fragment is isolated from a 1.5% agarose gel and inserted into HindIII/SalI digested plasmid pUC18. Ligation is carried out as described above. Ligated DNA is transformed into *E. coli* XL1 Blue™ (Stratagene). Two recombinant plasmids are isolated and the insert DNA is sequenced as described above using pUC universal and reverse primers (Boehringer). One plasmid containing the expected product is designated pWW22 (SEQ ID NO:9) and used as a source for further sub-cloning steps. Plasmid pWW22 is cleaved with HindIII and SalI, the mutated ETA gene fragment is isolated, and inserted into the large fragment of HindIII/SalI digested plasmid pMS150A containing pUC9 vector sequences and part of the ETA gene coding for the C-terminal half of the toxin. Thereby in the resulting plasmid pWW20 a truncated ETA gene coding for domains II and III of the toxin is created.

**13.2 Assembly of single-chain Fv-ETA fusion genes:** HindIII/XbaI single-chain Fv gene fragments suitable for the construction of Fv-ETA fusion genes are isolated from plasmid pWW53 (single-chain Fv FRP5), and plasmid pWW15-Fv51-ORF (single-chain Fv FWP51) and inserted into HindIII/XbaI digested pWW20. Ligation and transformation into *E. coli* XL1 Blue™ (Stratagene) are carried out as described above. The resulting plasmids pWW20-Fv5 (Fv(FRP5)-ETA) and pWW20-Fv51 (Fv(FWP51)-ETA) are used as a source for further sub-cloning steps.

**13.3 Construction of single-chain Fv-exotoxin A fusion gene expression plasmids:** For the expression of single-chain Fv-exotoxin A fusion genes in *E. coli* the expression plasmid pFLAG-1 (IBI Biochemicals) is used. The fusion-genes are fused in frame to the outer membrane protein A (ompA) signal sequence encoded by pFLAG-1. Plasmid DNA from pWW20-Fv5 and pWW20-Fv51 is digested with HindIII and blunt ends are created by Klenow fill-in as described in Example 4.5. Blunt ended DNA is digested with EcoRI and single-chain Fv-ETA gene fragments are isolated (Fv(FRP5)-ETA: 1916 bp, Fv(FWP51)-ETA: 1916 bp). pFLAG-1 plasmid DNA is digested with HindIII, blunt ends are created as described above, the resulting DNA fragment is isolated, and digested with EcoRI. Blunt-end/EcoRI Fv-ETA fusion gene fragments are inserted into the modified pFLAG-1 plasmid DNA. Thereby Fv-ETA fragments are fused in frame to the ompA signal sequence of pFLAG-1 creating plasmids pWW215-5 for the expression of Fv(FRP5)-ETA (SEQ ID NO: 10) and pWW215-51 for the expression of Fv(FWP51)-ETA (SEQ ID NO: 11):

#### Example 14. Expression and isolation of Fv(FRP5)-ETA and Fv(FWP51)-ETA from *E. coli*

**14.1 Preparation of total lysates:** Plasmids pWW215-5 and pWW215-51 are transformed into the *E. coli* strain CC118 according to standard procedures (see Example 9.1). Single colonies are picked and grown overnight in LB medium containing 100 µg/ml ampicillin and 0.4% glucose. The overnight cultures are diluted 1:30 in fresh LB medium containing ampicillin and glucose and grown at 37°C to an OD<sub>550</sub> of 0.5. At this point expression of the Fv(FRP5)-ETA and Fv(FWP51)-ETA genes is induced by the addition of IPTG to a final concentration of 0.5 mM, and the cells are grown for an additional 30 min. The cells are harvested by centrifugation and lysed by sonication in PBS/ 1 mM CaCl<sub>2</sub>. The lysates are cleared by ultracentrifugation at 25 000 g for 45 min at 4°C. The supernatants are collected.

14.2 Isolation of Fv(FRP5)-ETA and Fv(FWP51)-ETA by affinity chromatography: Cleared *E.coli* lysates containing the 66.4 kDa Fv(FRP5)-ETA or the 66.3 kDa Fv(FWP51)-ETA protein are passed through a M1 monoclonal antibody affinity column (IBI Biochemicals). The column is washed three times with PBS/1 mM CaCl<sub>2</sub>. Bound Fv(FRP5)-ETA or Fv(FWP51)-ETA proteins are eluted with PBS/2 mM EDTA. The fractions are monitored for the presence of Fv-ETA proteins by SDS-PAGE and immunoblotting (see Example 1.3.2) using an anti-exotoxin A antiserum developed in rabbit.

Example 15. Selective inhibition of protein synthesis in c-erbB-2 expressing cells with Fv(FRP5)-ETA and Fv(FWP51)-ETA

In vitro the recombinant immunotoxins Fv(FRP5)-ETA and Fv(FWP51)-ETA selectively inhibit protein synthesis and growth of cells expressing high levels of the human c-erbB-2 protein. The immunotoxins do not affect cells expressing no, or low levels of human c-erbB-2 protein.

15.1 Immunotoxin treatment of cell lines: Human breast and ovarian tumor cell lines SK-BR3, MDAMB-231, MDA-MB-453, HTB77, the mouse mammary epithelial cell line HC11, and HC11 cells transfected with the human c-erbB-2 cDNA are plated on 48 well tissue culture plates (Costar) at a density of 10<sup>5</sup> cells/well. After 4 h the medium is removed and replaced by normal growth medium containing Fv(FRP5)-ETA or Fv(FWP51)-ETA at various concentrations ranging from 1 to 1000 ng/ml. The cells are incubated with toxin fusion proteins for 16 h.

15.2 <sup>3</sup>H-leucine labeling of cells: The immunotoxin-treated cells are washed twice and incubated in normal growth medium containing 4  $\mu$ Ci <sup>3</sup>H-leucine/ml for 4 h. The labeled cells are washed twice and <sup>3</sup>H-leucine labeled total proteins are harvested by TCA precipitation onto Whatman GFC filters. The rate of protein synthesis in immunotoxin-treated cells is determined in comparison to untreated control cells.

Example 16: Fv(FRP5)-ETA and MAbs FWP51 and FSP77 inhibit the growth of c-erbB-2 expressing cells in nude mice.

The administration of Fv(FRP5)-ETA and the MAbs FWP51 and FSP77 to animals injected with c-erbB-2 expressing cells inhibits the tumor growth of these cells.

16.1 Nude mouse tumor model: The NIH/3T3 mouse fibroblast cell line is transfected according to conventional, previously described methods (Graham & van der Eb, Virology 52: 456, 1973) with a plasmid expressing the point mutated, activated human c-erbB-2 protein (Masuko et al., Jpn. Cancer Res. 80: 10, 1989) and with the plasmid pSV2neo (Southern & Berg, J. Mol. Appl. Genet. 1:327, 1982) which encodes the gene for resistance to the drug G418. Transfected cells are selected 2 weeks in medium containing 500  $\mu$ g/ml G418 (Geneticin, Gibco-BRL). Individual clones are selected and analyzed for the expression of the human c-erbB-2 protein using conventional protein blotting techniques (Towbin et al., Proc. Natl. Acad. Sci. USA 76: 4350, 1979). A clone expressing moderate levels of the point mutated, activated human c-erbB-2 protein (clone 3.7) is selected, and tested for growth in nude mice. 2-5 x 10<sup>6</sup> clone 3.7 cells (per animal) suspended in 0.2 ml PBS are subcutaneously injected into the flank of female Balb/c nude mice. The 3.7 cells injected at a dose of 2 x 10<sup>6</sup> cells rapidly form tumors in nude mice (control animals, cf. Example 16.2).

16.2 Immunotoxin treatment of animals: 2 x 10<sup>6</sup> clone 3.7 cells are injected subcutaneously into nude mice. The animals are treated continuously for a total of 7 days with the Fv(FRP5)-ETA. 200  $\mu$ l of Fv(FRP5)-ETA (concentration 35  $\mu$ g/ml in PBS) is placed in an osmotic pump (Alzet mini osmotic pump, Model 2001, Alza, Palo Alto, CA, #94303-0802) which is implanted subcutaneously into the animals at the same time as the clone 3.7 cells are injected. The pump continuously releases Fv(FRP5)-ETA and delivers 1  $\mu$ g/day for 7 days to each animal. In comparison with the control animals (cf. Example 16.1), the administration of Fv(FRP5)-ETA delays the onset of tumor formation.

16.3 MAB treatment of animals: 5 x 10<sup>6</sup> clone 3.7 cells are injected subcutaneously into nude mice. Starting on the same day as injection of clone 3.7 cells, the animals are treated daily, for a total of 10 days, with either MAb FWP51 or MAb FSP77 (MAB dose is 50  $\mu$ g/200  $\mu$ l BSS/day). The MAB is injected intravenously in the tail vein of the mouse. Both antibodies delay the onset of tumor growth. Compared therewith, a synergistic effect in inhibiting tumor growth is observed on simultaneous administration of both antibodies MAB FWP51 and MAB FSP77.

Sequence listing

5 SEQ ID NO:1

SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 361 bp  
 10 MOLECULE TYPE: plasmid DNA  
 ORIGINAL SOURCE ORGANISM: mouse  
 IMMEDIATE EXPERIMENTAL SOURCE: E. coli  
 NAME OF CELL CLONE: pMZ16/1

15 FEATURES: from 6 to 27 bp VH1BACK primer region  
 from 95 to 109 bp CDR<sub>1H</sub>  
 from 152 to 202 bp CDR<sub>2H</sub>  
 from 299 to 328 bp CDR<sub>3H</sub>  
 from 329 to 361 bp VH1FOR primer region

20 PROPERTIES: encodes the heavy chain variable domain of monoclonal antibody FRP5

25 TCTAGAGGTG AAAGTGCAGC AGTCTGGACC TGAAGTGAAG AAGCCTGGAG 50

AGACAGTCAA GATCTCCTGC AAGGCCTCTG GGTATCCTTT CACAAACTAT 100

30 GGAATGAACT GGGTGAAGCA GGCTCCAGGA CAGGGTTTAA AGTGGATGGG 150

CTGGATTAAC ACCTCCACTG GAGAGTCAAC ATTTGCTGAT GACTTCAAGG 200

35 GACGGTTTGA CTTCTCTTTG GAAACCTCTG CCAACACTGC CTATTTGCAG 250

ATCAACAACC TCAAAAGTGA AGACATGGCT ACATATTTCT GTGCAAGATG 300

40 GGAGGTTTAC CACGGCTACG TTCCTTACTG GGGCCAAGGG ACCACGGTCA 350

45 CCGTCTCCTC A 361

50

55

SEQ ID NO:2

5 SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 407 bp  
 MOLECULE TYPE: plasmid DNA  
 ORIGINAL SOURCE ORGANISM: mouse  
 IMMEDIATE EXPERIMENTAL SOURCE: E. coli  
 10 NAME OF CELL CLONE: pMZ18/1

15 FEATURES: from 6 to 28 bp MCK2 primer region  
 from 98 to 130 bp CDR<sub>1L</sub>  
 from 176 to 196 bp CDR<sub>2L</sub>  
 from 293 to 319 bp CDR<sub>3L</sub>  
 from 374 to 404 bp MCK2 primer region

PROPERTIES: encodes the kappa light chain variable domain of monoclonal antibody FRP5

20

TCTAGTCACT GGATGGTGGG AAGATGGAGA CATTGTGATG ACCCAGTCTC 50

25

ACAAATTCCT GTCCACTTCA GTAGGAGACA GGGTCAGCAT CACCTGCAAG 100

30

GCCAGTCAGG ATGTGTATAA TGCTGTTGCC TGGTATCAAC AGAAACCAGG 150

35

ACAATCTCCT AACTTCTGA TTTACTCGGC ATCCTCCCGG TACTGAGGAG 200

40

TCCCTTCTCG CTTCCTGAGC AGTGGCTCTG GGCCGGATTT CACTTTCACC 250

ATCAGCAGTG TGCAGGCTGA AGACCTGGCA GTTTATTCTCT GTCAGCAACA 300

45

TTTTTCGTACT CCATTCACGT TCGGCTCGGG GACAAAATTG GAAATAAAAC 350

GGGCTGATGC TGCACCAACT GATCCATCT TCCCACCATC CAGTGACTAG 400

50

AACTAGA 407

55

55

## SEQ ID NO:3

5 SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 175 bp  
 MOLECULE TYPE: plasmid DNA  
 ORIGINAL SOURCE ORGANISM: fully synthetic  
 IMMEDIATE EXPERIMENTAL SOURCE: E. coli  
 10 NAME OF CELL CLONE: pWW19

15 FEATURES: from 30 to 35 bp PstI site  
 from 38 to 44 bp BstEII site for subcloning of heavy chain  
 variable domain  
 from 54 to 98 bp coding sequence of (GlyGlyGlyGlySer)<sub>3</sub>  
 linker  
 from 105 to 110 bp PvuII site  
 from 112 to 117 bp BglII site  
 from 120 to 125 bp BclI site for subcloning of light chain variable  
 20 domain

25 AAGCTTGCAT GCAAGCTTCT CAGGTACAAC TGCAGGAGGT CACCGTTTCC 50  
 TCTGGCGGTG GCGGTTCTGG TGGCGGTGGC TCCGGCGGTG GCGGTTCTGA 100  
 30 CATCCAGCTG GAGATCTAGC TGATCAAAGC TCTAGAGGAT CCCCGGGTAC 150  
 CGAGCTCGAA TTCACTGGCC GTCGT 175

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SEQ ID NO:4

5 SEQUENCE TYPE: nucleotide with corresponding protein  
 SEQUENCE LENGTH: 748 bp  
 MOLECULE TYPE: plasmid DNA  
 ORIGINAL SOURCE ORGANISM: mouse  
 IMMEDIATE EXPERIMENTAL SOURCE: E. coli  
 10 NAME OF CELL CLONE: pWW52

FEATURES: from 1 to 8 bp synthetic spacer  
 from 9 to 365 bp FRP5 heavy chain variable domain  
 from 99 to 113 bp CDR<sub>1H</sub>  
 15 from 156 to 206 bp CDR<sub>2H</sub>  
 from 303 to 332 bp CDR<sub>3H</sub>  
 from 366 to 410 bp 15 amino acids linker sequence  
 from 411 to 728 bp FRP5 light chain variable domain  
 from 480 to 512 bp CDR<sub>1L</sub>  
 20 from 558 to 578 bp CDR<sub>2L</sub>  
 from 675 to 701 bp CDR<sub>3L</sub>

PROPERTIES: Fv heavy chain/light chain variable domain fusion protein binding to the  
 extracellular domain of the  
 growth factor receptor c-erbB-2  
 25

30 AAGCT TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG 41  
 Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu  
 5 10

35 AAG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC TCT 83  
 Lys Lys Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser  
 15 20 25

40 GGG TAT CCT TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG 125  
 Gly Tyr Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys Gln  
 30 35 40

45 GCT CCA GGA CAG GGT TTA AAG TGG ATG GGC TGG ATTAAC ACT 167  
 Ala Pro Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn Thr  
 45 50

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5 TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA CGG 209  
 Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly Arg  
 55 60 65

10 TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT TTG 251  
 Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu  
 70 75 80

15 CAG ATC AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC 293  
 Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe  
 85 90 95

20 TGT GCA AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG 335  
 Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp  
 100 105 110

25 GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT 377  
 Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly  
 115 120

30 TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG 419  
 Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln  
 125 130 135

35 CTG ACC CAG TCT CAC AAA TTC CTG TCC ACT TCA GTA GGA GAC 461  
 Leu Thr Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly Asp  
 140 145 150

40 AGG GTC AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT AAT 503  
 Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr Asn  
 155 160 165

45 GCT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT AAA 545  
 Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys  
 170 175 180

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	CTT CTG ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC CCT	587
	Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val Pro	
5	185 190	
	TCT CGC TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT TTC	629
10	Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe	
	195 200 205	
	ACC ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC	671
15	Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe	
	210 215 220	
	TGT CAG CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG	713
20	Cys Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly	
	225 230 235	
	ACA AAA TTG GAG ATC TAGCTGATCA AAGCTCTAGA	748
25	Thr Lys Leu Glu Ile	
	240	

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SEQ ID NO:5

5

SEQUENCE TYPE: nucleotide with corresponding protein

SEQUENCE LENGTH: 2233 bp

MOLECULE TYPE: plasmid DNA

ORIGINAL SOURCE ORGANISM: mouse and E. coli

10

IMMEDIATE EXPERIMENTAL SOURCE: E. coli

NAME OF CELL CLONE: pWW616

15

FEATURES: from 1 to 22 bp

from 23 to 85 bp

from 89 to 445 bp

from 446 to 490 bp

from 491 to 814 bp

from 815 to 2155 bp

from 2156 to 2233 bp

ompA 5' non-coding region

ompA signal peptide

FRP5 heavy chain variable domain

15 amino acids linker sequence

FRP5 light chain variable domain

coding region of phoA

3' non-coding region of phoA

20

PROPERTIES: Fv heavy chain/light chain variable domain and alkaline phosphatase  
fusion protein Fv(FRP5)-phoA binding to the growth factor receptor c-erbB-2

25

TCTAGATAAC GAGGCGCAAA AA

ATG AAA AAG ACA GCT ATC GCG

43

Met Lys Lys Thr Ala Ile Ala

-20

-15

30

ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAA GCT

85

Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala

-10

-5

35

TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA CTG AAG AAG

127

Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys

1

5

10

40

CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC TCT GGG TAT

169

Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr

15

20

25

45

CCT TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA

211

Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro

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5	GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT AAC ACC TCC ACT	253
	Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn Thr Ser Thr	
	45 50 55	
10	GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA CGG TTT GAC	295
	Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly Arg Phe Asp	
	60 65 70	
15	TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT TTG CAG ATC	337
	Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu Gln Ile	
	75 80	
20	AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT TTC TGT GCA	379
	Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr Phe Cys Ala	
	85 90 95	
25	AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC TGG GGC CAA	421
	Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr Trp Gly Gln	
	100 105 110	
30	GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT TCT GGT	463
	Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly	
	115 120 125	
35	GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG CTG ACC	505
	Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr	
	130 135 140	
40	CAG TCT CAC AAA TTC CTG TCC ACT TCA GTA GGA GAC AGG GTC	547
	Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly Asp Arg Val	
	145 150	
45	AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT AAT GCT GTT	589
	Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr Asn Ala Val	
50	155 160 165	

55

5	GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT AAA CTT CTG Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu 170 175 180	631
10	ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC CCT TCT CGC Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val Pro Ser Arg 185 190 195	673
15	TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT TTC ACC ATC Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr Phe Thr Ile 200 205 210	715
20	AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Phe Cys Gln 215 220	757
25	CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG GGG ACA AAA Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser Gly Thr Lys 225 230 235	799
30	TTG GAG ATC AAA GCT CTA GAG CCT GTT CTG GAA AAC CGG GCT Leu Glu Ile Lys Ala Leu Glu Pro Val Leu Glu Asn Arg Ala 240 245 250	841
35	GCT CAG GGC GAT ATT ACT GCA CCC GGC GGT GCT CGC CGT TTA Ala Gln Gly Asp Ile Thr Ala Pro Gly Gly Ala Arg Arg Leu 255 260 265	883
40	ACG GGT GAT CAG ACT GCC GCT CTG CGT GAT TCT CTT AGC GAT Thr Gly Asp Gln Thr Ala Ala Leu Arg Asp Ser Leu Ser Asp 270 275 280	925
45	AAA CCT GCA AAA AAT ATT ATT TTG CTG ATT GGC GAT GGG ATG Lys Pro Ala Lys Asn Ile Ile Leu Leu Ile Gly Asp Gly Met 285 290	967

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5 GGG GAC TCG GAA ATT ACT GCC GCA CGT AAT TAT GCC GAA GGT 1009  
 Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr Ala Glu Gly  
 295 300 305

10 GCG GGC GGC TTT TTT AAA GGT ATA GAT GCC TTA CCG CTT ACC 1051  
 Ala Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu Thr  
 310 315 320

15 GGG CAA TAC ACT CAC TAT GCG CTG AAT AAA AAA ACC GGC AAA 1093  
 Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys  
 325 330 335

20 CCG GAC TAC GTC ACC GAC TCG GCT GCA TCA GCA ACC GCC TGG 1135  
 Pro Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp  
 340 345 350

25 TCA ACC GGT GTC AAA ACC TAT AAC GGC GCG CTG GGC GTC GAT 1177  
 Ser Thr Gly Val Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp  
 355 360

30 ATT CAC GAA AAA GAT CAC CCA ACG ATT CTG GAA ATG GCA AAA 1219  
 Ile His Glu Lys Asp His Pro Thr Ile Leu Glu Met Ala Lys  
 365 370 375

35 GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT ACC GCA GAG TTG 1261  
 Ala Ala Gly Leu Ala Thr Gly Asn Val Ser Thr Ala Glu Leu  
 380 385 390

40 CAG GAT GCC ACG CCC GCT GCG CTG GTG GCA CAT GTG ACC TCG 1303  
 Gln Asp Ala Thr Pro Ala Ala Leu Val Ala His Val Thr Ser  
 395 400 405

45 CGC AAA TGC TAC GGT CCG AGC GCG ACC AGT GAA AAA TGT CCG 1345  
 Arg Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu Lys Cys Pro  
 410 415 420

50  
 55

5 GGT AAC GCT CTG GAA AAA GGC GGA AAA GGA TCG ATT ACC GAA 1387  
 Gly Asn Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr Glu  
 425 430

10 CAG CTG CTT AAC GCT CGT GCC GAC GTT ACG CTT GGC GGC GGC 1429  
 Gln Leu Leu Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly  
 435 440 445

15 GCA AAA ACC TTT GCT GAA ACG GCA ACC GCT GGT GAA TGG CAG 1471  
 Ala Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln  
 450 455 460

20 GGA AAA ACG CTG CGT GAA CAG GCA CAG GCG CGT GGT TAT CAG 1513  
 Gly Lys Thr Leu Arg Glu Gln Ala Gln Ala Arg Gly Tyr Gln  
 465 470 475

25 TTG GTG AGC GAT GCT GCC TCA CTG AAT TCG GTG ACG GAA GCG 1555  
 Leu Val Ser Asp Ala Ala Ser Leu Asn Ser Val Thr Glu Ala  
 480 485 490

30 AAT CAG CAA AAA CCC CTG CTT GGC CTG TTT GCT GAC GGC AAT 1597  
 Asn Gln Gln Lys Pro Leu Leu Gly Leu Phe Ala Asp Gly Asn  
 495 500

35 ATG CCA GTG CGC TGG CTA GGA CCG AAA GCA ACG TAC CAT GGC 1639  
 Met Pro Val Arg Trp Leu Gly Pro Lys Ala Thr Tyr His Gly  
 505 510 515

40 AAT ATC GAT AAG CCC GCA GTC ACC TGT ACG CCA AAT CCG CAA 1681  
 Asn Ile Asp Lys Pro Ala Val Thr Cys Thr Pro Asn Pro Gln  
 520 525 530

45 CGT AAT GAC AGT GTA CCA ACC CTG GCG CAG ATG ACC GAC AAA 1723  
 Arg Asn Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp Lys  
 535 540 545

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5       GCC ATT GAA TTG TTG AGT AAA AAT GAG AAA GGC TTT TTC CTG 1765  
       Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu  
                   550                       555                       560

10       CAA GTT GAA GGT GCG TCA ATC GAT AAA CAG GAT CAT GCT GCG 1807  
       Gln Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His Ala Ala  
                   565                       570

15       AAT CCT TGT GGG CAA ATT GGC GAG ACG GTC GAT CTC GAT GAA 1849  
       Asn Pro Cys Gly Gln Ile Gly Glu Thr Val Asp Leu Asp Glu  
       575                       580                       585

20       GCC GTA CAA CGG GCG CTG GAA TTC GCT AAA AAG GAG GGT AAC 1891  
       Ala Val Gln Arg Ala Leu Glu Phe Ala Lys Lys Glu Gly Asn  
                   590                       595                       600

25       ACG CTG GTC ATA GTC ACC GCT GAT CAC GCC CAC GCC AGC CAG 1933  
       Thr Leu Val Ile Val Thr Ala Asp His Ala His Ala Ser Gln  
                   605                       610                       615

30       ATT GTT GCG CCG GAT ACC AAA GCT CCG GGC CTC ACC CAG GCG 1975  
       Ile Val Ala Pro Asp Thr Lys Ala Pro Gly Leu Thr Gln Ala  
                   620                       625                       630

35       CTA AAT ACC AAA GAT GGC GCA GTG ATG GTG ATG AGT TAC GGG 2017  
       Leu Asn Thr Lys Asp Gly Ala Val Met Val Met Ser Tyr Gly  
                   635                       640

40       AAC TCC GAA GAG GAT TCA CAA GAA CAT ACC GGC AGT CAG TTG 2059  
       Asn Ser Glu Glu Asp Ser Gln Glu His Thr Gly Ser Gln Leu  
       645                       650                       655

45       CGT ATT GCG GCG TAT GGC CCG CAT GCC GCC AAT GTT GTT GGA 2101  
       Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn Val Val Gly  
       660                       665                       670

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EP 0 502 812 A1

CTG ACC GAC CAG ACC GAT CTC TTC TAC ACC ATG AAA GCC GCT 2143  
 Leu Thr Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys Ala Ala  
 675 680 685

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CTG GGG CTG AAA TAAAACCGCG CCCGGCAGTG AATTTTCGCT 2185  
 Leu Gly Leu Lys  
 690

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GCCGGGTGGT TTTTGTGCTG TTAGCAACCA GACTTAATGG CAGAGCTC 2233

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SEQ ID NO:6

5 SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 342 bp  
 MOLECULE TYPE: plasmid DNA  
 ORIGINAL SOURCE ORGANISM: mouse  
 10 IMMEDIATE EXPERIMENTAL SOURCE: E.coli  
 NAME OF CELL CLONE: pWW15-VH51-1

15 FEATURES: from 1 to 14 bp partial sequence of VH1BACK primer region  
 from 82 to 96 bp CDR<sub>1H</sub>  
 from 139 to 189 bp CDR<sub>2H</sub>  
 from 286 to 318 bp CDR<sub>3H</sub>  
 20 from 317 to 342 bp partial sequence of VH1FOR primer region

PROPERTIES: encodes the heavy chain variable domain of monoclonal antibody FWP51  
 25

CTGCAGCAGT CTGGGGCTGA GCTGGTGAGG CCTGGGACTT CAGTGAAGCT 50  
 30 GTCCTGCAAG GCTTCTGATT ACACCTTCAC CAGCTACTGG ATGAACTGGG 100  
 TGAAGCAGAG GCCTGGACAA GGCCTTGAAT GGATTGGTAT GATTGATCCT 150  
 35 TCAGACAGTG AAACTCAATA CAATCAAATG TTCAAGGACA AGGCCGCATT 200  
 GACTGTAGAC AAGTCCTCCA ATACAGCCTA CATGCAACTC AGCAGCCTGA 250  
 40 CATCTGAGGA CTCTGCGGTC TATTACTGTG CAAAAGGGGG GGCCTCTGGG 300  
 GACTGGTACT TCGATGTCTG GGGCCAAGGG ACCACGGTCA CC 342  
 45

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SEQ ID NO:7

5 SEQUENCE TYPE: nucleotide  
 SEQUENCE LENGTH: 310 bp  
 MOLECULE TYPE: plasmid DNA  
 ORIGINAL SOURCE ORGANISM: mouse  
 10 IMMEDIATE EXPERIMENTAL SOURCE: E.coli  
 NAME OF CELL CLONE: pWW15-VL51-1

15 FEATURES: from 1 to 18 bp partial sequence of VK1BACK primer region  
 from 64 to 96 bp CDR<sub>1L</sub>  
 from 142 to 162 bp CDR<sub>2L</sub>  
 from 259 to 282 bp CDR<sub>3L</sub>  
 20 from 292 to 310 bp partial sequence of VK1FOR primer region

25 PROPERTIES: encodes the light chain variable domain of monoclonal antibody FWP51

CAGCTGACCC AGTCTCCATC CTCACTGTCT GCATCTCTGG GAGGCGAAGT 50  
 30 CACCATCACT TGCAAGGCAA GCCAAGACAT TAAGAAGTAT ATAGCTTGGT 100  
 ACCAACACAA GCCTGGAAAA AGTCCTCGGC TACTCATACA CTACACATCT 150  
 35 GTATTACAGC CAGGCATCCC ATCCAGGTTC AGTGGAAGTG GGTCTGGGAG 200  
 AGATTATTCC TTCAGCATCC ACAACCTGGA GCCTGAAGAT ATTGCAACTT 250  
 40 ATTATTGTCT ACATTATGAT TATCTGTACA CGTTCGGAGG GGGCACCAAG 300  
 45 CTGGAGATCT 310

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SEQ ID NO:8

5

SEQUENCE TYPE: nucleotide with corresponding protein

SEQUENCE LENGTH: 748 bp

MOLECULE TYPE: plasmid DNA ORIGINAL

10

SOURCE ORGANISM: mouse

IMMEDIATE EXPERIMENTAL SOURCE: E.coli

NAME OF CELL CLONE: pWW15-Fv51

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FEATURES:	from 1 to 8 bp	synthetic spacer
	from 9 to 368 bp	FWP 51 heavy chain variable domain
	from 99 to 113 bp	CDR1H
20	from 156 to 206 bp	CDR2H
	from 303 to 335 bp	CDR3H
	from 369 to 413 bp	synthetic spacer
	from 414 to 728 bp	FWP 51 light chain variable domain
25	from 483 to 515 bp	CDR1L
	from 561 to 581 bp	R2L
	from 678 to 701 bp	CDR3L
30	from 729 to 748 bp	synthetic spacer

PROPERTIES: encodes single-chain Fv fusion gene comprising monoclonal antibody  
FWP51 heavy and kappa light chain variable domain cDNA

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AAGCT

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TCT CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG CTG GTG  
Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Val  
1 5 10

44

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AGG CCT GGG ACT TCA GTG AAG CTG TCC TGC AAG GCT TCT GAT  
Arg Pro Gly Thr Ser Val Lys Leu Ser Cys Lys Ala Ser Asp  
15 20 25

86

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5      TAC ACC TTC ACC AGC TAC TGG ATG AAC TGG GTG AAG CAG AGG 128  
       Tyr Thr Phe Thr Ser Tyr Trp Met Asn Trp Val Lys Gln Arg  
               30                                35                                40

10      CCT GGA CAA GGC CTT GAA TGG ATT GGT ATG ATT GAT CCT TCA 170  
       Pro Gly Gln Gly Leu Glu Trp Ile Gly Met Ile Asp Pro Ser  
               45                                50                                55

15      GAC AGT GAA ACT CAA TAC AAT CAA ATG TTC AAG GAC AAG GCC 212  
       Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe Lys Asp Lys Ala  
               60                                65

20      GCA TTG ACT GTA GAC AAG TCC TCC AAT ACA GCC TAC ATG CAA 254  
       Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr Met Gln  
               70                                75                                80

25      CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT TAC TGT 296  
       Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys  
               85                                90                                95

30      GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT GTC TGG 338  
       Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp Val Trp  
               100                                105                                110

35      GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC GGT 380  
       Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly  
               115                                120                                125

40      TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC CAG 422  
       Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln  
               130                                135

45      CTG ACC CAG TCT CCA TCC TCA CTG TCT GCA TCT CTG GGA GGC 464  
       Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Leu Gly Gly  
               145                                150

55

5 GAA GTC ACC ATC ACT TGC AAG GCA AGC CAA GAC ATT AAG AAG 506  
 Glu Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Lys 168  
 155 160 165

10 TAT ATA GCT TGG TAC CAA CAC AAG CCT GGA AAA AGT CCT CGG 548  
 Tyr Ile Ala Trp Tyr Gln His Lys Pro Gly Lys Ser Pro Arg 182  
 170 175 180

15 CTA CTC ATA CAC TAC ACA TCT GTA TTA CAG CCA GGC ATC CCA 590  
 Leu Leu Ile His Tyr Thr Ser Val Leu Gln Pro Gly Ile Pro  
 185 190 195

20 TCC AGG TTC AGT GGA AGT GGG TCT GGG AGA GAT TAT TCC TTC 632  
 Ser Arg Phe Ser Gly Ser Gly Ser Gly Arg Asp Tyr Ser Phe  
 200 205

25 AGC ATC CAC AAC CTG GAG CCT GAA GAT ATT GCA ACT TAT TAT 674  
 Ser Ile His Asn Leu Glu Pro Glu Asp Ile Ala Thr Tyr Tyr  
 210 215 220

30 TGT CTA CAT TAT GAT TAT CTG TAC ACG TTC GGA GGG GGC ACC 716  
 Cys Leu His Tyr Asp Tyr Leu Tyr Thr Phe Gly Gly Gly Thr  
 225 230 235

35 AAG CTG GAG ATC TAGCTGATCA AAGCTCTAGA 748  
 Lys Leu Glu Ile  
 40 240

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15	<b>FEATURES:</b> from 1 to 27 bp from 29 to 201 bp	synthetic spacer partial exotoxin A sequence corresponding to nucleotide positions 1574 to 1747 bp of the exotoxin A sequence (Gray et al., Proc. Natl. Acad. Sci. USA 81: 2645, 1984)
20		

AAGCTTAAGG	AGATCTGCAT	GCTTCTAGAG	GGCGGCAGCC	TGGCCGCGCT	50
GACCGCGCAC	CAGGCCTGCC	ACCTGCCGCT	GGAGACTTTC	ACCCGTCATC	100
GCCAGCCGCG	CGGCTGGGAA	CAACTGGAGC	AGTGCGGCTA	TCCGGTGCAG	150
CGGCTGGTCG	CCCTCTACCT	GGCGGCGCGA	CTGTCATGGA	ACCAGGTCGA	200
C					201

SEQ ID NO:10

5 SEQUENCE TYPE: nucleotide with corresponding protein  
 SEQUENCE LENGTH: 2012 bp  
 MOLECULE TYPE: plasmid DNA  
 10 ORIGINAL SOURCE ORGANISM: mouse/P.aeruginosa  
 IMMEDIATE EXPERIMENTAL SOURCE: E.coli  
 NAME OF CELL CLONE: pWW215-5

15 FEATURES: from 1 to 63 bp ompA signal peptide  
 from 64 to 87 bp FLAG peptide and enterokinase cleavage  
 site  
 20 from 97 to 453 bp FRP5 heavy chain variable domain  
 from 454 to 498 bp 15 amino acids linker sequence  
 from 499 to 822 bp FRP5 light chain variable domain  
 from 826 to 1911 bp exotoxin A gene coding region  
 25 (coding for amino acids 252 to 613 of the  
 mature exotoxin A)  
 from 1912 to 2012 bp 3'non-coding region of the exotoxin A  
 gene

30

PROPERTIES: Fv heavy chain/light chain variable domain and exotoxin A fusion protein  
 Fv(FRP5)-ETA binding to the c-erbB-2 protein

35

ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT 42  
 Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly  
 40 -30 -25 -20

TTC GCT ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC 84  
 Phe Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp  
 45 -15 -10 -5

AAG CTA GCT TCT CAG GTA CAA CTG CAG CAG TCT GGA CCT GAA 126  
 50 Lys Leu Ala Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu  
 1 5 10

55

	CTG AAG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GCC	168
	Leu Lys Lys Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala	
5	15 20 25	
	TCT GGG TAT CCT TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG	210
10	Ser Gly Tyr Pro Phe Thr Asn Tyr Gly Met Asn Trp Val Lys	
	30 35	
	CAG GCT CCA GGA CAG GGT TTA AAG TGG ATG GGC TGG ATT AAC	252
15	Gln Ala Pro Gly Gln Gly Leu Lys Trp Met Gly Trp Ile Asn	
	40 45 50	
	ACC TCC ACT GGA GAG TCA ACA TTT GCT GAT GAC TTC AAG GGA	294
20	Thr Ser Thr Gly Glu Ser Thr Phe Ala Asp Asp Phe Lys Gly	
	55 60 65	
	CGG TTT GAC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT	336
25	Arg Phe Asp Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr	
	70 75 80	
	TTG CAG ATC AAC AAC CTC AAA AGT GAA GAC ATG GCT ACA TAT	378
30	Leu Gln Ile Asn Asn Leu Lys Ser Glu Asp Met Ala Thr Tyr	
	85 90 95	
35		
	TTC TGT GCA AGA TGG GAG GTT TAC CAC GGC TAC GTT CCT TAC	420
	Phe Cys Ala Arg Trp Glu Val Tyr His Gly Tyr Val Pro Tyr	
40	100 105	
	TGG GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT GGC	462
	Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly	
45	110 115 120	
	GGT TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC ATC	504
50	Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile	
	125 130 135	

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CAG CTG ACC CAG TCT CAC AAA TTC CTG TCC ACT TCA GTA GGA 546  
 Gln Leu Thr Gln Ser His Lys Phe Leu Ser Thr Ser Val Gly  
 5                   140                   145                   150

GAC AGG GTC AGC ATC ACC TGC AAG GCC AGT CAG GAT GTG TAT 588  
 Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Tyr  
 10                   155                   160                   165

AAT GCT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT 630  
 Asn Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro  
 15                   170                   175

AAA CTT CTG ATT TAC TCG GCA TCC TCC CGG TAC ACT GGA GTC 672  
 Lys Leu Leu Ile Tyr Ser Ala Ser Ser Arg Tyr Thr Gly Val  
 20                   180                   185                   190

CCT TCT CGC TTC ACT GGC AGT GGC TCT GGG CCG GAT TTC ACT 714  
 Pro Ser Arg Phe Thr Gly Ser Gly Ser Gly Pro Asp Phe Thr  
 25                   195                   200                   205

TTC ACC ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GTT TAT 756  
 Phe Thr Ile Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr  
 30                   210                   215                   220

TTC TGT CAG CAA CAT TTT CGT ACT CCA TTC ACG TTC GGC TCG 798  
 Phe Cys Gln Gln His Phe Arg Thr Pro Phe Thr Phe Gly Ser  
 35                   225                   230                   235

GGG ACA AAA TTG GAG ATC AAA GCT CTA GAG GGC GGC AGC CTG 840  
 Gly Thr Lys Leu Glu Ile Lys Ala Leu Glu Gly Gly Ser Leu  
 40                   240                   245

GCC GCG CTG ACC GCG CAC CAG GCC TGC CAC CTG CCG CTG GAG 882  
 Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu  
 45                   250                   255                   260

55

ACT TTC ACC CGT CAT CGC CAG CCG CGC GGC TGG GAA CAA CTG 924  
 Thr Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu  
 5           265                               270                               275

GAG CAG TGC GGC TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC 966  
 10   Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr  
           280                               285                               290

CTG GCG GCG CGA CTG TCA TGG AAC CAG GTC GAC CAG GTG ATC 1008  
 15   Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile  
                   295                               300                               305

CGC AAC GCC CTG GCC AGC CCC GGC AGC GGC GGC GAC CTG GGC 1050  
 20   Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly  
                   310                               315

GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC CGT CTG GCC CTG 1092  
 25   Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu  
       320                               325                               330

ACC CTG GCC GCC GCC GAG AGC GAG CGC TTC GTC CGG CAG GGC 1134  
 30   Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly  
           335                               340                               345

ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG GTG 1176  
 35   Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val  
                   350                               355                               360

AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG 1218  
 40   Ser Leu Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro  
                   365                               370                               375

GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC AAC TAT CCC ACT 1260  
 50   Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr  
                   380                               385

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5 GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC AGC 1302  
 Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser  
 390 395 400

10 ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG 1344  
 Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln  
 405 410 415

15 GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC 1386  
 Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly  
 420 425 430

20 TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC 1428  
 Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe  
 435 440 445

25 GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG 1470  
 Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp  
 450 455

30 CGC GGT TTC TAT ATC GCC GGC GAT CCG GCG CTG GCC TAC GGC 1512  
 Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly  
 460 465 470

35 TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC CGG ATC CGC 1554  
 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg  
 475 480 485

40 AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG 1596  
 Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu  
 490 495 500

45 CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG 1638  
 Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu  
 505 510 515

55

5 GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG 1680  
 Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro  
 520 525

10 CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG 1722  
 Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly  
 530 535 540

15 CGC CTG GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC 1764  
 Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr  
 545 550 555

20 GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC 1806  
 Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val  
 560 565 570

25 GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1848  
 Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln  
 575 580 585

30 GCG ATC AGC GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA 1890  
 Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys  
 590 595

35 CCG CCG CGC GAG GAC CTG AAG TAA CTGCCGCGAC CGGCCGGCTC 1934  
 Pro Pro Arg Glu Asp Leu Lys  
 600 605

40 CCTTCGCAGG AGCCGGCCTT CTCGGGGCCT GGCCATACAT CAGGTTTTTC 1984  
 TGATGCCAGC CCAATCGAAT ATGAATTC 2012

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 50  
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SEQ ID NO:11

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SEQUENCE TYPE: nucleotide with corresponding protein

SEQUENCE LENGTH: 2012 bp

MOLECULE TYPE: plasmid DNA

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ORIGINAL SOURCE ORGANISM: mouse/*P.aeruginosa*IMMEDIATE EXPERIMENTAL SOURCE: *E.coli*

NAME OF CELL CLONE: pWW215-51

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FEATURES:	from 1 to 63 bp	ompA signal peptide
	from 64 to 87 bp	FLAG peptide and enterokinase cleavage site
20	from 97 to 456 bp	FWP51 heavy chain variable domain
	from 457 to 501 bp	15 amino acids linker sequence
	from 502 to 822 bp	FWP51 light chain variable domain
25	from 826 to 1911 bp	exotoxin A gene coding region (coding for amino acids 252 to 613 of the mature exotoxin A)
	from 1912 to 2012 bp	3' non-coding region of the exotoxin A gene

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PROPERTIES: Fv heavy chain/light chain variable domain and exotoxin A fusion protein  
Fv(FWP51)-ETA binding to the c-crbB-2 protein

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ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT	42
Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly	
-30 -25 -20	

40

TTC GCT ACC GTT GCG CAA GCT GAC TAC AAG GAC GAC GAT GAC	84
Phe Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Asp	
-15 -10 -5	

45

AAG CTA GCT TCT CAG GTA CAA CTG CAG CAG TCT GGG GCT GAG	126
Lys Leu Ala Ser Gln Val Gln Leu Gln Gln Ser Gly Ala Glu	
1 5 10	

50

55

CTG GTG AGG CCT GGG ACT TCA GTG AAG CTG TCC TGC AAG GCT 168  
 Leu Val Arg Pro Gly Thr Ser Val Lys Leu Ser Cys Lys Ala  
 5 15 20 25

TCT GAT TAC ACC TTC ACC AGC TAC TGG ATG AAC TGG GTG AAG 210  
 Ser Asp Tyr Thr Phe Thr Ser Tyr Trp Met Asn Trp Val Lys  
 10 30 35

CAG AGG CCT GGA CAA GGC CTT GAA TGG ATT GGT ATG ATT GAT 252  
 Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile Gly Met Ile Asp  
 15 40 45 50

CCT TCA GAC AGT GAA ACT CAA TAC AAT CAA ATG TTC AAG GAC 294  
 Pro Ser Asp Ser Glu Thr Gln Tyr Asn Gln Met Phe Lys Asp  
 20 55 60 65

AAG GCC GCA TTG ACT GTA GAC AAG TCC TCC AAT ACA GCC TAC 336  
 Lys Ala Ala Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr  
 25 70 75 80

ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCG GTC TAT 378  
 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr  
 30 85 90 95

TAC TGT GCA AAA GGG GGG GCC TCT GGG GAC TGG TAC TTC GAT 420  
 Tyr Cys Ala Lys Gly Gly Ala Ser Gly Asp Trp Tyr Phe Asp  
 35 100 105

GTC TGG GGC CAA GGG ACC ACG GTC ACC GTT TCC TCT GGC GGT 462  
 Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly  
 40 110 115 120

GGC GGT TCT GGT GGC GGT GGC TCC GGC GGT GGC GGT TCT GAC 504  
 Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp  
 45 125 130 135

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ACT TTC ACC CGT CAT CGC CAG CCG CGC GGC TGG GAA CAA CTG 924  
 Thr Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu  
 5           265                               270                               275

GAG CAG TGC GGC TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC 966  
 10   Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr  
           280                               285                               290

CTG GCG GCG CGA CTG TCA TGG AAC CAG GTC GAC CAG GTG ATC 1008  
 15   Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val Ile  
                   295                               300                               305

CGC AAC GCC CTG GCC AGC CCC GGC AGC GGC GGC GAC CTG GGC 1050  
 20   Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly  
                   310                               315

GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC CGT CTG GCC CTG 1092  
 25   Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu  
   320                               325                               330

ACC CTG GCC GCC GCC GAG AGC GAG CGC TTC GTC CGG CAG GGC 1134  
 30   Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly  
           335                               340                               345

ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG GTG 1176  
 35   Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val  
           350                               355                               360

AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG 1218  
 40   Ser Leu Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro  
 45                   365                               370                               375

GCG GAC AGC GGC GAC GCC CTG CTG GAG CGC AAC TAT CCC ACT 1260  
 50   Ala Asp Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr  
                   380                               385

55



5 GGC GCG GAG TTC CTC GGC GAC GGC GGC GAC GTC AGC TTC AGC 1302  
 Gly Ala Glu Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser  
 390 395 400

10 ACC CGC GGC ACG CAG AAC TGG ACG GTG GAG CGG CTG CTC CAG 1344  
 Thr Arg Gly Thr Gln Asn Trp Thr Val Glu Arg Leu Leu Gln 448  
 405 410 415

15 GCG CAC CGC CAA CTG GAG GAG CGC GGC TAT GTG TTC GTC GGC 1386  
 Ala His Arg Gln Leu Glu Glu Arg Gly Tyr Val Phe Val Gly  
 420 425 430

20 TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA AGC ATC GTC TTC 1428  
 Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile Val Phe  
 435 440 445

25 GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG ATC TGG 1470  
 Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp  
 450 455

30 CGC GGT TTC TAT ATC GCC GGC GAT CCG GCG CTG GCC TAC GGC 1512  
 Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly  
 460 465 470

35 TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC CGG ATC CGC 1554  
 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg  
 475 480 485

40 AAC GGT GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG 1596  
 Asn Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu  
 490 495 500

45 CCG GGC TTC TAC CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG 1638  
 Pro Gly Phe Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu  
 505 510 515

55

5 GCG GCG GGC GAG GTC GAA CGG CTG ATC GGC CAT CCG CTG CCG 1680  
 Ala Ala Gly Glu Val Glu Arg Leu Ile Gly His Pro Leu Pro  
 520 525

10 CTG CGC CTG GAC GCC ATC ACC GGC CCC GAG GAG GAA GGC GGG 1722  
 Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu Glu Glu Gly Gly  
 530 535 540

15 CGC CTG GAG ACC ATT CTC GGC TGG CCG CTG GCC GAG CGC ACC 1764  
 Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu Arg Thr  
 545 550 555

20 GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC AAC GTC 1806  
 Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val  
 560 565 570

25 GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1848  
 Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln  
 575 580 585

30 GCG ATC AGC GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA 1890  
 Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys  
 590 595

35 CCG CCG CGC GAG GAC CTG AAG TAA CTGCCGCGAC CGGCCGGCTC 1934  
 Pro Pro Arg Glu Asp Leu Lys  
 600 605

40 CCTTCGCAGG AGCCGGCCTT CTCGGGGCCT GGCCATACAT CAGGTTTTTC 1984

45 TGATGCCAGC CCAATCGAAT ATGAATTC 2012

# 50 Claims

1. A recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody.
- 55 2. A recombinant antibody according to claim 1 wherein the heavy chain variable domain comprises a polypeptid of th formula
 
$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \quad (I)$$
 wherein  $FR_1$  is a polypeptide residue comprising 25-33 naturally occurring amino acids,  $FR_2$  is a polyp p-

5 tide residue comprising 12-16 naturally occurring amino acids, FR<sub>3</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>4</sub> is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR<sub>1H</sub> is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4, CDR<sub>2H</sub> is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4, and CDR<sub>3H</sub> is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

10 3. A recombinant antibody according to claim 2 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR<sub>1</sub>), 37 to 50 (FR<sub>2</sub>), 68 to 99 (FR<sub>3</sub>), and/or 110 to 120 (FR<sub>4</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

15 4. A recombinant antibody according to claim 2 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

20 5. A recombinant antibody according to claim 1 wherein the light chain variable domain comprises a polypeptide of the formula

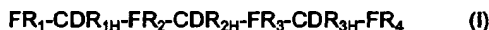


20 wherein FR<sub>6</sub> is a polypeptide residue comprising naturally occurring amino acids, FR<sub>7</sub> is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR<sub>8</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>9</sub> is a polypeptide residue comprising naturally occurring amino acids, CDR<sub>1L</sub> is a polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4, CDR<sub>2L</sub> is a polypeptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4, and CDR<sub>3L</sub> is a polypeptide residue of the amino acid sequence 224 to 232 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

25 6. A recombinant antibody according to claim 5 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein optionally one or more single amino acids within the amino acid sequences 136 to 158 (FR<sub>6</sub>), 170 to 184 (FR<sub>7</sub>), 192 to 223 (FR<sub>8</sub>), and/or 233 to 241 (FR<sub>9</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

30 7. A recombinant antibody according to claim 5 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

35 8. A recombinant antibody according to claim 1 wherein the heavy chain variable domain comprises a polypeptide of the formula



40 wherein FR<sub>1</sub> is a polypeptide residue comprising 25-33 naturally occurring amino acids, FR<sub>2</sub> is a polypeptide residue comprising 12-16 naturally occurring amino acids, FR<sub>3</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>4</sub> is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR<sub>1H</sub> is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:8, CDR<sub>2H</sub> is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:8, and CDR<sub>3H</sub> is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

45 9. A recombinant antibody according to claim 8 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR<sub>1</sub>), 37 to 50 (FR<sub>2</sub>), 68 to 99 (FR<sub>3</sub>), and/or 111 to 121 (FR<sub>4</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

50 10. A recombinant antibody according to claim 8 wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

11. A recombinant antibody according to claim 1 wherein the light chain variable domain comprises a polypeptide of the formula

$$FR_6-CDR_{1L}-FR_7-CDR_{2L}-FR_8-CDR_{3L}-FR_9 \quad (II)$$

wherein  $FR_6$  is a polypeptide residue comprising naturally occurring amino acids,  $FR_7$  is a polypeptide residue comprising 13-17 naturally occurring amino acids,  $FR_8$  is a polypeptide residue comprising 30-34 naturally occurring amino acids,  $FR_9$  is a polypeptide residue comprising naturally occurring amino acids,  $CDR_{1L}$  is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:8,  $CDR_{2L}$  is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO: 8, and  $CDR_{3L}$  is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

12. A recombinant antibody according to claim 11 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO: 8, wherein optionally one or more single amino acids within the amino acid sequences 137 to 159 ( $FR_6$ ), 171 to 185 ( $FR_7$ ), 193 to 224 ( $FR_8$ ), and/or 233 to 241 ( $FR_9$ ) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

13. A recombinant antibody according to claim 11 wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

14. A recombinant antibody according to claim 1 which is a chimeric antibody consisting of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human heavy chain constant domain  $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  or  $\mu$ , and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain  $\kappa$  or  $\lambda$ , all assembled to give a functional antibody.

15. A recombinant antibody according to claim 1 which is a single-chain antibody wherein the heavy chain variable domain and the light chain variable domain are linked by a polypeptide spacer group.

16. A single-chain recombinant antibody according to claim 15 further comprising an effector molecule and optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer.

17. A single-chain recombinant antibody according to claim 16 wherein the effector molecule is an enzyme or a biologically active variant thereof.

18. A single-chain recombinant antibody according to claim 16 wherein the enzyme is alkaline phosphatase or a biologically active variant thereof.

19. A single-chain recombinant antibody according to claim 16 wherein the effector molecule is a toxin or a biologically active variant thereof.

20. A single-chain recombinant antibody according to claim 19 wherein the effector molecule is Pseudomonas exotoxin or a biologically active variant thereof.

21. A single-chain recombinant antibody according to claim 16 wherein the heavy chain variable domain and the light chain variable domain are derived from a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77.

22. A single-chain recombinant antibody according to claim 21, further comprising an effector molecule or a biologically active variant thereof.

23. A single-chain recombinant antibody according to claim 21 wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FRP5.

24. A single chain recombinant antibody according to claim 21 wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FWP51.

25. A single-chain recombinant antibody according to claim 22 comprising the heavy chain variable domain of the mouse monoclonal antibody FRP5, the 15 amino acid polypeptide consisting of three repetitive sub-units of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FRP5, and

an enzyme or a toxin, or a biologically active variant thereof.

26. A single-chain recombinant antibody designated Fv(FRP5)-phoA according to claim 25 comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO:5.
27. A single-chain recombinant antibody designated Fv(FRP5)-ETA according to claim 25 comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 10.
28. A single-chain recombinant antibody according to claim 22 comprising the heavy chain variable domain of the mouse monoclonal antibody FWP51, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FWP51, and an enzyme or a toxin, or a biologically active variant thereof.
29. A single-chain recombinant antibody designated Fv(FWP51)-ETA according to claim 28 comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 11.
30. A mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 selected from the group consisting of antibodies designated FRP5, FSP16, FSP77 and FWP51.
31. A monoclonal antibody according to claim 30 designated FRP5.
32. A monoclonal antibody according to claim 30 designated or FWP51.
33. A method of manufacture of a recombinant antibody according to claim 1 or of a monoclonal antibody according to claim 30, characterized in that cells producing such an antibody are multiplied in vitro or in vivo and, when required, the obtained antibody is isolated.
34. A hybridoma cell secreting a monoclonal antibody according to claims 30.
35. A process for the preparation of a hybridoma cell line according to claim 34 secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2, characterized in that a suitable mammal is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected.
36. A recombinant DNA comprising an insert coding for a recombinant antibody according to claim 1.
37. A recombinant DNA according to claim 36 comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said heavy chain variable domain.
38. A recombinant DNA according to claim 36 comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said light chain variable domain.
39. A recombinant DNA according to claim 36 which is a hybrid vector further comprising an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.
40. A hybrid vector according to claim 39 comprising a Simian virus promoter and the mouse Ig H or L chain enhancer.
41. A process for the preparation of a DNA according to claim 36 comprising the steps of
  - a) preparing murine DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity,
  - b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule,
  - c) synthesizing DNA coding for the desired spacer group by chemical methods,
  - d) constructing recombinant genes encoding the recombinant antibodies by incorporating the DNA of

st p a) and, ptionally, b) and/or c) into appropriate hybrid vectors,  
 ) transf rring the obtain d hybrid vectors into a recipi nt host cell or retri ving th DNA coding for th  
 recombinant g nes and transferring th unlinked DNA into a recipient host cell,  
 f) selecting and culturing th transformed host cell, and  
 g) optionally isolating the desired DNA.

42. A host cell transformed with a recombinant DNA according to claim 36.
43. A host cell according to claim 42 which is a cell of a strain of E. coli.
44. A process for the preparation of a transformed host cell according to claim 42 wherein suitable recipient cells are transformed with a hybrid vector comprising a DNA insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of an antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites, and the transformed cells are selected.
45. Use of a recombinant antibody according to claim 1 for the qualitative and quantitative determination of the growth factor receptor c-erbB-2.
46. Use of a monoclonal antibody according to claim 30 for the qualitative and quantitative determination of the growth factor receptor c-erbB-2.
47. Use according to claim 45 comprising immunostaining of tissue sections with a solution containing the recombinant antibody comprising a detectable enzyme.
48. A test kit for the qualitative and quantitative determination of c-erbB-2 protein comprising a recombinant antibody according to claim 1 and/or a monoclonal antibody according to claim 30.
49. A recombinant antibody according to claim 1 for use in the treatment of the human or animal body.
50. A monoclonal antibody according to claim 30 for use in the treatment of the human or animal body.
51. A pharmaceutical composition for treating tumors over-expressing the growth factor receptor c-erbB-2 comprising a therapeutically effective amount of a recombinant antibody according to claim 1 or of a monoclonal antibody according to claim 30 and a pharmaceutically acceptable carrier.
52. The use of a recombinant antibody according to claim 1 and/or a monoclonal antibody according to claim 30 for the manufacture of a pharmaceutical preparation.

#### Claims for the following Contracting State : ES

1. Process for the preparation of a recombinant antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 comprising a heavy chain variable domain and a light chain variable domain of a monoclonal antibody characterized in that cells producing such an antibody are multiplied in vitro or in vivo and, when required, the obtained antibody is isolated.
2. Process according to claim 1 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the formula
 
$$FR_1\text{-}CDR_{1H}\text{-}FR_2\text{-}CDR_{2H}\text{-}FR_3\text{-}CDR_{3H}\text{-}FR_4 \quad (I)$$
 wherein  $FR_1$  is a polypeptide residue comprising 25-33 naturally occurring amino acids,  $FR_2$  is a polypeptide residue comprising 12-16 naturally occurring amino acids,  $FR_3$  is a polypeptide residue comprising 30-34 naturally occurring amino acids,  $FR_4$  is a polypeptide residue comprising 6-13 naturally occurring amino acids,  $CDR_{1H}$  is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:4,  $CDR_{2H}$  is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:4, and  $CDR_{3H}$  is a polypeptide residue of the amino acid sequence 100 to 109 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.
3. Process according to claim 2 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein

optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR<sub>1</sub>), 37 to 50 (FR<sub>2</sub>), 68 to 99 (FR<sub>3</sub>), and/or 110 to 120 (FR<sub>4</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 5 4. Process according to claim 2 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 120 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 10 5. Process according to claim 1 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the formula

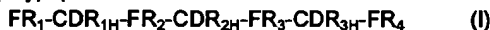


wherein FR<sub>6</sub> is a polypeptide residue comprising naturally occurring amino acids, FR<sub>7</sub> is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR<sub>8</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>9</sub> is a polypeptide residue comprising naturally occurring amino acids, CDR<sub>1L</sub> is a polypeptide residue of the amino acid sequence 159 to 169 of SEQ ID NO:4, CDR<sub>2L</sub> is a polypeptide residue of the amino acid sequence 185 to 191 of SEQ ID NO:4, and CDR<sub>3L</sub> is a polypeptide residue of the amino acid sequence 224 to 232 of SEQ ID NO:4, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 20 6. Process according to claim 5 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein optionally one or more single amino acids within the amino acid sequences 136 to 158 (FR<sub>6</sub>), 170 to 184 (FR<sub>7</sub>), 192 to 223 (FR<sub>8</sub>), and/or 233 to 241 (FR<sub>9</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 25 7. Process according to claim 5 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 136 to 241 of SEQ ID NO:4, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 30 8. Process according to claim 1 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the formula



wherein FR<sub>1</sub> is a polypeptide residue comprising 25-33 naturally occurring amino acids, FR<sub>2</sub> is a polypeptide residue comprising 12-16 naturally occurring amino acids, FR<sub>3</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>4</sub> is a polypeptide residue comprising 6-13 naturally occurring amino acids, CDR<sub>1H</sub> is a polypeptide residue of the amino acid sequence 32 to 36 of SEQ ID NO:8, CDR<sub>2H</sub> is a polypeptide residue of the amino acid sequence 51 to 67 of SEQ ID NO:8, and CDR<sub>3H</sub> is a polypeptide residue of the amino acid sequence 100 to 110 of SEQ ID NO:8, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 40 9. Process according to claim 8 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein optionally one or more single amino acids within the amino acid sequences 2 to 31 (FR<sub>1</sub>), 37 to 50 (FR<sub>2</sub>), 68 to 99 (FR<sub>3</sub>), and/or 111 to 121 (FR<sub>4</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 45 10. Process according to claim 8 for the preparation of a recombinant antibody wherein the heavy chain variable domain comprises a polypeptide of the amino acid sequence 2 to 121 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.

- 50 11. Process according to claim 1 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the formula



wherein FR<sub>6</sub> is a polypeptide residue comprising naturally occurring amino acids, FR<sub>7</sub> is a polypeptide residue comprising 13-17 naturally occurring amino acids, FR<sub>8</sub> is a polypeptide residue comprising 30-34 naturally occurring amino acids, FR<sub>9</sub> is a polypeptide residue comprising naturally occurring amino acids, CDR<sub>1L</sub> is a polypeptide residue of the amino acid sequence 160 to 170 of SEQ ID NO:8, CDR<sub>2L</sub> is a polypeptide residue of the amino acid sequence 186 to 192 of SEQ ID NO:8, and CDR<sub>3L</sub> is a polypeptide residue of the amino acid sequence 225 to 232 of SEQ ID NO:8, and wherein the amino acid Cys may be

in the oxidized state forming S-S-bridges.

12. Process according to claim 11 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein optionally one or more single amino acids within the amino acid sequences 137 to 159 (FR<sub>6</sub>), 171 to 185 (FR<sub>7</sub>), 193 to 224 (FR<sub>8</sub>), and/or 233 to 241 (FR<sub>9</sub>) are replaced by other amino acids or deleted, and wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.
13. Process according to claim 11 for the preparation of a recombinant antibody wherein the light chain variable domain comprises a polypeptide of the amino acid sequence 137 to 241 of SEQ ID NO:8, wherein the amino acid Cys may be in the oxidized state forming S-S-bridges.
14. Process according to claim 1 for the preparation of a recombinant antibody which is a chimeric antibody consisting of a mouse heavy chain variable domain with the specificity for c-erbB-2 and a human heavy chain constant domain  $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  or  $\mu$ , and of a mouse light chain variable domain with the specificity for c-erbB-2 and a human light chain constant domain  $\kappa$  or  $\lambda$ , all assembled to give a functional antibody.
15. Process according to claim 1 for the preparation of a recombinant antibody which is a single-chain antibody wherein the heavy chain variable domain and the light chain variable domain are linked by a polypeptide spacer group.
16. Process according to claim 15 for the preparation of a single-chain recombinant antibody further comprising an effector molecule and optionally comprising a peptide facilitating purification, a cleavage site and a peptide spacer.
17. Process according to claim 16 for the preparation of a single-chain recombinant antibody wherein the effector molecule is an enzyme or a biologically active variant thereof.
18. Process according to claim 16 for the preparation of a single-chain recombinant antibody wherein the enzyme is alkaline phosphatase or a biologically active variant thereof.
19. Process according to claim 16 for the preparation of a single-chain recombinant antibody wherein the effector molecule is a toxin or a biologically active variant thereof.
20. Process according to claim 19 for the preparation of a single-chain recombinant antibody wherein the effector molecule is Pseudomonas exotoxin or a biologically active variant thereof.
21. Process according to claim 19 for the preparation of a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derived from a mouse monoclonal antibody selected from the group consisting of FRP5, FSP16, FWP51 and FSP77.
22. Process according to claim 21 for the preparation of a single-chain recombinant antibody, further comprising an effector molecule or a biologically active variant thereof.
23. Process according to claim 21 for the preparation of a single-chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FRP5.
24. Process according to claim 21 for the preparation of a single chain recombinant antibody wherein the heavy chain variable domain and the light chain variable domain are derived from the mouse monoclonal antibody FWP51.
25. Process according to claim 22 for the preparation of a single-chain recombinant antibody comprising the heavy chain variable domain of the mouse monoclonal antibody FRP5, the 15 amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FRP5, and an enzyme or a toxin, or a biologically active variant thereof.
26. Process according to claim 25 for the preparation of a single-chain recombinant antibody designated Fv(FRP5)-phoA comprising a polypeptide of the amino acid sequence 2 to 690 of SEQ ID NO:5.



27. Process according to claim 25 for the preparation of a single-chain recombinant antibody designated Fv(FRP5)-ETA comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 10.
28. Process according to claim 22 for the preparation of a single-chain recombinant antibody comprising the heavy chain variable domain of the mouse monoclonal antibody FWP51, the amino acid polypeptide consisting of three repetitive subunits of Gly-Gly-Gly-Gly-Ser, the light chain variable domain of the mouse monoclonal antibody FWP51, and an enzyme or a toxin, or a biologically active variant thereof.
29. Process according to claim 28 for the preparation of a single-chain recombinant antibody designated Fv(FWP51)-ETA comprising a polypeptide of the amino acid sequence 2 to 606 of SEQ ID NO: 11.
30. Process for the preparation of a mouse monoclonal antibody directed to the extracellular domain of the growth factor receptor c-erbB-2 selected from the group consisting of antibodies designated FRP5, FSP16, FSP77 and FWP51 characterized in that cells producing such an antibody are multiplied in vitro or in vivo and, when required, the obtained antibody is isolated.
31. A process according to claim 30 for the preparation of a monoclonal antibody designated FRP5.
32. A process according to claim 30 for the preparation of a monoclonal antibody designated or FWP51.
33. A process for the preparation of a hybridoma cell line secreting monoclonal antibodies directed to the extracellular domain of the growth factor receptor c-erbB-2 selected from the group consisting of antibodies designated FRP5, FSP16, FSP77 and FWP51, characterized in that a suitable mammal is immunized with purified c-erbB-2 protein, an antigenic carrier containing purified c-erbB-2 or with cells bearing c-erbB-2, antibody-producing cells of the immunized mammal are fused with cells of a suitable myeloma cell line, the hybrid cells obtained in the fusion are cloned, and cell clones secreting the desired antibodies are selected.
34. A process for the preparation of a DNA comprising an insert coding for a recombinant antibody obtainable according to claim 1 comprising the steps of
  - a) preparing murine DNA from the genome of a suitable hybridoma cell line and selecting the desired DNA coding for the variable heavy and/or light chain domains of the antibody with the desired specificity,
  - b) preparing DNA coding for the desired signal sequence and/or preparing DNA coding for an effector molecule,
  - c) synthesizing DNA coding for the desired spacer group by chemical methods,
  - d) constructing recombinant genes encoding the recombinant antibodies by incorporating the DNA of step a) and, optionally, b) and/or c) into appropriate hybrid vectors,
  - e) transferring the obtained hybrid vectors into a recipient host cell or retrieving the DNA coding for the recombinant genes and transferring the unlinked DNA into a recipient host cell,
  - f) selecting and culturing the transformed host cell, and
  - g) optionally isolating the desired DNA.
35. A process according to claim 34 for the preparation of a DNA comprising an insert coding for a heavy chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said heavy chain variable domain.
36. A process according to claim 34 for the preparation of a recombinant DNA comprising an insert coding for a light chain murine variable domain of a monoclonal antibody selected from the group consisting of antibodies FRP5, FSP16, FSP77 and FWP51 or coding for an amino acid sequence homologous to said light chain variable domain.
37. A process according to claim 34 for the preparation of a recombinant DNA which is a hybrid vector further comprising an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.
38. A process according to claim 34 for the preparation of hybrid vector comprising a Simian virus promoter and the mouse Ig H or L chain enhancer.

39. A process for the preparation of a host cell transformed with a recombinant DNA obtainable according to claim 34 wherein suitable recipient cells are transformed with a hybrid vector comprising a DNA insert coding for a heavy chain murine variable domain and/or for a light chain murine variable domain of an antibody directed to the extracellular domain of the growth factor receptor c-erbB-2, an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites, and the transformed cells are selected.
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40. A process for the preparation of a host cell according to claim 42 wherein the host cell is a cell strain of E. coli.
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European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 92 81 0056

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.5)
X,D	WO-A-8 906 692 (GENENTECH, INC) 27 July 1989 * the whole document *	1, 45, 47, 49, 51, 52	C12N15/13 C12P21/08 C12N1/21 G01N33/574 A61K39/395
X	WO-A-9 014 357 (GENENTECH, INC.) 29 November 1990 * the whole document *	1, 45, 47, 49, 51, 52	
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, no. 21, 1 November 1990, WASHINGTON US pages 8291 - 8295; R.J. KREITMAN ET AL.; 'The recombinant immunotoxin anti-Tac(Fv)-Pseudomonas exotoxin 40 is' * the whole document *	15-29	
A,D	WO-A-8 911 533 (THE UNITED STATES OF AMERICA) 30 November 1989 * example 1 *	15-29	
A	SCIENCE, vol. 240, 20 May 1988, LANCASTER, PA US pages 1038 - 1041; A. SKERRA AND A. PLUCKTHUN; 'Assembly of a functional immunoglobulin Fv fragment in Escherichia coli' * figure 1 *	18	TECHNICAL FIELDS SEARCHED (Int. CL.5)  C07K C12N C12P G01N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 07 MAY 1992	Examiner CUPIDO M.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons * : member of the same patent family, corresponding document	

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